ACROSOME SIZE AND KINEMATICS OF HUMAN SPERMATOZOA

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DECLARATION

I, the undersigned, hereby declare that the work in this thesis is my own original work and that I have not previously, in its entirety or in part, submitted it at any university for a degree.

Signature: .....

Date: .....

ABSTRACT

For spermatozoa to gain access to the oocyte for fertilization, lytic enzymes need to be released during the acrosome reaction. These enzymes, which are stored and transported within an organelle termed the acrosome, make it possible for spermatozoa to collectively penetrate the layers of cells and glycoproteins that surround and protect an oocyte. Acrosomes may thus be viewed as essential for fertilization and their shape, size and volume were examined morphometrically by utilizing automated morphometric analysis equipment.

In addition to the acrosome being necessary for normal unassisted fertilization, spermatozoa also need the ability to migrate to the oocyte. Following *zona pellucida* binding, sperm tail thrust movement initiates *zona* penetration into the space created by the digestive action of the acrosomal enzymes. Therefore the motion characteristics of spermatozoa were also quantified in terms of kinematic properties.

In the treatment of male sub fertility, assisted reproductive techniques are applied. In the application of such techniques, a motile sub-population of spermatozoa was obtained by employing a procedure (swim-up selection) that selects cells on the basis of their kinematic ability.

This study presents an analysis of the morphometric and kinematic qualities of spermatozoa populations that are subjected to swim-up selection and investigates the relationship of these morphometrical and kinematic qualities.

Computer-assisted semen analysis, swim-up selection and automated sperm morphology analysis tests were all used to evaluate spermatozoa populations. Results indicated that, irrespective of acrosome size, higher kinematic parameter measurements were observed post-swim-up. A significant inverse relationship between the population's average acrosome size and a number of kinematic parameters was observed.

Our results indicated that for a post-swim-up population of spermatozoa an increase in the average acrosome size was significantly related to a decrease in the kinematic parameters VAP, VCL and the VSL within the same population.

OPSOMMING

Vir spermatozoa om toegang te verkry tot die oösiet, ten einde fertilisasie te bewerkstellig, word lietiese ensieme deur midel van die akrosoomreaksie vrygestel. Hierdie ensieme word in die organelle wat as die akrosoom bekend staan geberg, en wanneer vrygelaat, maak hierdie ensieme dit moontlik vir spermatozoa om deur die lae selle sowel as die glikoproteine, wat die oösiet omring en beskerm, te dring. Die akrosoom word dus beskou as noodsaaklik vir normale bevrugting. Met behulp van analitiese metodes, wat kinematiese en morfometriese parameters kwantitatief evalueer, is dit moontlik om die bewegings patrone sowel as die akrosomale vorm, groote en volume van ’n bevolking spermatozoa te ondersoek. Een benadering vir die akkurate meting van akrosomale strukture behels die gebruik van ge-outomatiseerde sperm-morfologie analiserings toerusting.

Bykomend tot die noodsaaklike rol wat die akrosoom in natuurlike befrugting speel is dit nodig dat spermatozoa oor die vermoë beskik om deur die vroulike genitale stelsel tot by die oosiet te migreer. Verder is dit noodsaaklik om die *zona pellucida* te penetreer met behulp van akrosomale ensieme en stert bewegings. Die bewegings aksies van spermatozoa word gekwantifiseer en as kinematiese parameters beskryf.

`n Beweeglike sub-populasie spermatozoa word geisoleer vir ge-assisteerde reproduktiewe tegnieke in die behandeling van sub-fertiliteit. Hierdie sub-populasie word geselekteer deur van ’n prosedure (op-swem seleksie) gebruik te maak wat spermatozoa op grond van verskillende vlakke van kinematiese aktiwiteit skei.

Hierdie studie lê ’n analise van morfometriese en kinematiese kwaliteite van spermpopulasies wat onderwerp is aan op-swem seleksie voor, en ondersoek die verwantskappe tussen die morfometriese en kinematiese parameters.

Rekenaar-berekende semenanalise, akrosoomreaksie bepaling, op-swem seleksie, geoutomatiseerde spermmorfologie analise en in enkele gevalle *zona pellucida*-bindingtoetse was gebruik om spermatozoa groepe te evalueer. Resulate het getoon dat ongeag die akrosoomgrootte is hoër motiliteitsparameters waargeneem nadat die op-swem tegniek toegepas is. ‘n Beduidende omgekeerde verwantskap tussen die groep se gemiddelde akrosoomgrootte en verskeie bewegingsparameters is waargeneem, met toenemende gemiddelde akrosoomgrootte gekorreleer met afnemende motiliteitsparameters.

Ter afsluiting dui die resultate aan dat die gemiddelde akrosoom grootte van spermatozoa in ‘n op-swem groep, beduidend verwant is aan VAP, VCL en VSL.

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LIST OF ABBREVIATIONS

ASMA	automated sperm morphology / morphometry analysis
AI	acrosomal index refers to the percentage of spermatozoa with normal acrosomes within a population
ALH	amplitude of lateral head displacement
AR	acrosome reaction
AS	acrosome size
BCF	beat cross f nucleic acid
HZI	hemi*zona* index
ICSI	intracytoplasmic sperm injection
IVF	*in vitro* fertilization
IVOS	integrated visual optical system
KD	kilo Dalton
HTF	human tubal fluid
LIN	linearity
P	progesterone
PBS	phosphate buffered saline
PZD	partial *zona* dissection
rpm	revolutions per minute
SE	standard error
SUZI	sub-zonal insemination

STR	straightness
VAP	average path velocity
VCL	curvilinear velocity
VSL	straight line velocity
WHO	World Health Organisation
ZP	*zona pellucida*

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CHAPTER 1
INTRODUCTION

1.1 BACKGROUND

In order for normal fertilization to occur spermatozoa must be equipped with functional cellular structures that make the journey to, and penetration into, the ova possible. The movement of spermatozoa from their site of ejaculation to the site of fertilization is accomplished in part through flagellar motility[1]. This motion could be described by means of a number of characteristics that have been known to be significantly related to fertilization potential and conception *in vivo*[2].

On contact with the oocyte, receptor-dependent sperm-oocyte binding occurs and the acrosome releases its acrosomal contents in order to promote penetration beyond the cells and hyaluronic acid matrix obscuring the oocyte[3]. The acrosome is vital for *in vivo* (natural) fertilization, as well as for *in vitro* assisted reproductive technologies[4] with the exception of microsurgical fertilization techniques such as intracytoplasmic sperm injection (ICSI), partial *zona* dissection (PZD) and sub-zonal insemination (SUZI). Adequate kinematics and a functionally normal acrosome, to supply the needed enzymes, play an important role in successful natural fertilization[5].

1.2 THE ACROSOME

1.2.1 Acrosome formation

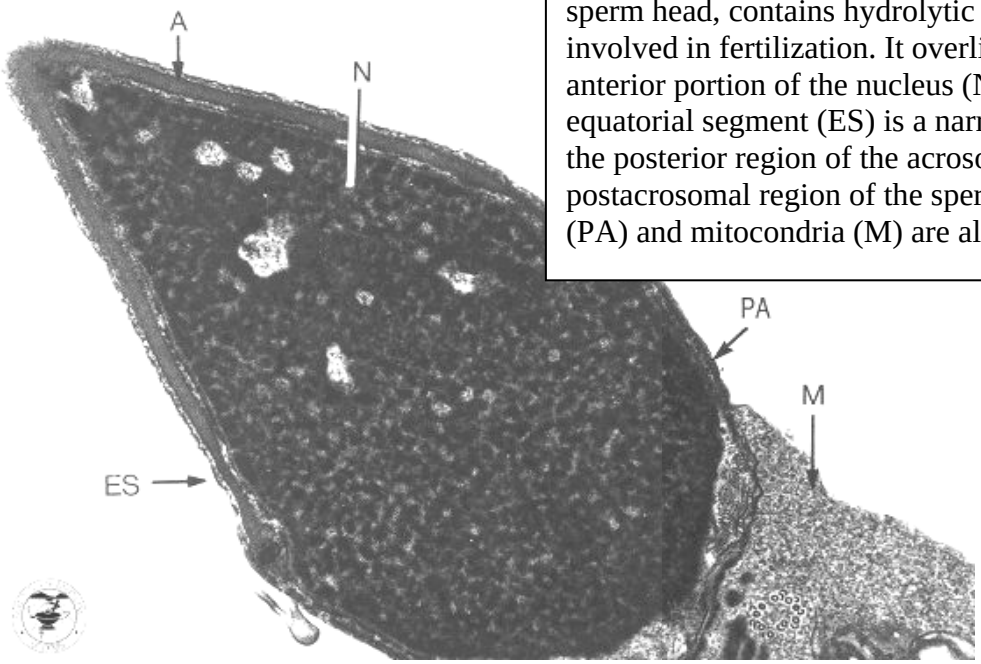
Male gamete formation (spermatogenesis) occurs in the seminiferous tubules of the testes. During embryological development, the primordial germ cells migrate from the yolk sac to the germinal ridge. These germ cells together with the somatic Sertoli cells form the foetal testes and seminiferous tubules which begin production of spermatozoa during puberty. Spermatogenesis could be divided into two phases namely, spermiogenesis and spermiation. It is during the post-meiotic development phase known as spermiogenesis that the developing spermatid undergoes characteristic morphological changes. During the subsequent spermiation phase, the spermatid gets released from the surrounding Sertoli cells by the severing of the cytoplasmic chords and enters the lumen of the seminiferous tubule. It is now referred to as a spermatozoon[6].

During mammalian spermiogenesis, six developmental stages of the acrosome could be seen. In stage I, round spermatid, proacrosomal granules fuse and attach to the assembled peri-nuclear theca to form the acrosomal vesicle[7]. The acrosomal matrix of mature spermatozoa originates from the acrosomal granule contained within this acrosomal vesicle[8]. A stage II spermatid displays a more rounded nucleus and better developed acrosome due to formation of the nuclear, inner acrosomal and outer acrosomal membranes (see Figure 1 and Figure 2). The acrosomal cap and plasma membrane become visible in a stage III spermatid. Hereafter, the acrosome increases in size as the Golgi apparatus buds and releases vesicles which fuse with the acrosomal membranes. Upon completion of acrosomal protein production, the Golgi apparatus separates from the acrosomal vesicle of elongating spermatids[9]. The

spermatid subsequently develops through stages IV to VI during which nuclear condensation and cytoplasm shedding occurs prior to spermiation[10].

1.2.2 Histology and anatomy

The acrosome is a secretary granule common to all mammals with great variation in the shape, size and enzymatic content being observed[11]. Apical organelles, located close to the nucleus, were already depicted by Leeuwenhoek in 1677[12] during his pioneering studies in microscopic cytology[13]. It was not, however, until the late nineteenth century that the acrosomal structures were finally recognized and interpreted[14].

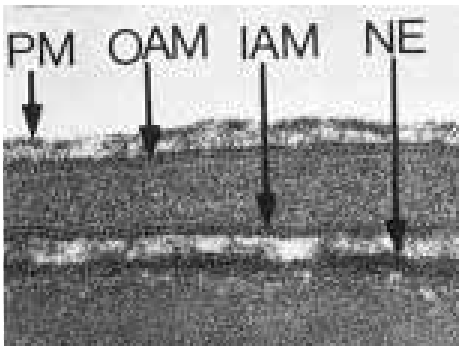


The acrosome (A), an organelle in the sperm head, contains hydrolytic enzymes involved in fertilization. It overlies the anterior portion of the nucleus (N). The equatorial segment (ES) is a narrowing of the posterior region of the acrosome. The postacrosomal region of the sperm head (PA) and mitocondria (M) are also seen.

Figure 1 Electron micrograph of the sperm head and acrosomal cap[15].

Microscopically, the acrosome appears as a thick cap-like layer of uniform thickness over the anterior region of the nucleus and is separated from the nuclear envelope by a gap within which filamentous material is discerned near the apex. In the area

posterior to the equatorial segment, the acrosomal membrane is pentalaminar and consists of hexagonally packed 20 nm (diameter of 2 x 8-10 nm) particles displaying geometrical arrangement.[16,17]



The outer acrosomal membrane (OAM) lies below and adjacent to the plasma membrane (PM), while the inner acrosomal membrane (IAM) lies adjacent to the nuclear envelope (NE).

Figure 2 Electron micrograph of acrosomal membranes[18].

Mammalian acrosome formation is characterised by the fusion of Golgi apparatus derived proacrosomal vesicles thereby forming a single lysosomic body, the acrosome, enveloped in a Golgi membrane, the acrosomal membrane. The inside of the acrosome acts as a storage site for the rich repertoire of acrosomal enzymes[19]. The only significant carbohydrate structural component within the acrosomal matrix, tubulin, is present in an equal distribution to acrosin, one of the acrosomal enzymes, suggesting that tubulin is the binding site for the proteinase[20].

The caudal region of the acrosome was believed to undergo final modification during the passage of the spermatozoa through the epididymis[21], this theory is now questioned[22]. The storage of the spermatozoa in the epididymis, where scrotal temperatures are low, results in enhanced oxygen solubility and decreased spermatozoa metabolism[23]. Although seen in some mammals, post-testicular acrosomal modification during passage through the post epididymal ducts is not seen in humans[24].

The acrosome was recognized as an essential part of spermatozoa morphology as seen in early observations made by Retzius[14]. According to the World Health Organisation (WHO) criteria of 1992, for spermatozoa to be classified as morphologically normal one requirement was that the acrosomal size must comprise more than 33% of the distal part of the sperm head[25]. More recently the strict criteria recommended by the revised 1999 WHO laboratory manual required the acrosomal size to cover 40-70% of the distal part of the sperm head[26] In addition the physical size of the acrosome was also an important criterion in predicting fertilization potential[27], the acrosome of morphologically normal spermatozoa thus varies in length from 1.80-3.85 µm and in width from 2.5-3.5 µm.

1.2.3 Capacitation

Freshly ejaculated spermatozoa are not immediately capable of fertilization possibly since after spermiation, decapacitation factors bind to the spermatozoa[28]. These factors may be of epididymal or seminal origin[29] and were thought to posses the ability to temporarily inhibit the ability of the spermatozoa to fertilize in a rapid and significant manner, even when introduced to previously capacitated spermatozoa[30]. Capacitation had been suggested to encompass the removal of these decapacitation factors, subsequently restoring fertilizing ability, though there is evidence suggesting that the binding proteins are involved in storage rather than capacitation[31]. During capacitation, the outer acrosomal membrane was believed to undergo several conformational changes which render the spermatozoa capable of undergoing the acrosome reaction (AR)[19].

Natural capacitation takes five to seven hours and occurs during the progression of spermatozoa through the uterus and oviducts. Some of the events occurring during

capacitation include the loss of sperm sterols[32], altered distribution of phospholipids in the plasma membrane[33], loss of molecules from the cell surface[34], membrane hyperpolarization[35], production of reactive oxygen species[36], elevated concentrations of calcium and cAMP[37], protein phosphorylation[38] and increased intracellular pH[39]. After these processes capacitated spermatozoa display higher levels of kinematic activity and hyperactivation.

The acquisition of fertilizing ability (the process of capacitation) is dependent on suitable conditions existing in the extracellular environment immediately adjacent to the spermatozoa. Ionic composition had a definitive effect on the ability of the spermatozoa to undergo stimulation or inhibition of the AR[40]. A large contingent of ions participate in these complex changes that accompany the acquisition of fertilizing ability[41], with Ca^{2+} fluxes at the forefront of both capacitation and AR processes. For maximal response, millimolar concentrations of Ca^{2+} were required[42]. The pivotal role of Ca^{2+} was illustrated by its research applications. Treatment of spermatozoa with Ca^{2+} ionophore (A23187) enriched medium promotes rapid capacitation. If used carefully to minimise negative effects on kinematics, ionophore-treated cells were immediately highly fertile[43].

1.2.4 The acrosome reaction

The AR is an exocytotic process that spermatozoa undergo in order to acquire fertilization potential, this was well illustrated in patients with globozoospermia in which the absence of the acrosome and its contents resulted in “severely reduced capacity to bind to the *zona pellucida* and penetrate an oocyte normally”[44]. Internal modification of spermatozoa was necessary if their acrosomal state of enzyme storage was to be replaced by acrosomal exocytosis and for fertilization to

subsequently become possible. This internal modification may be triggered by the extracellular ionic environment in conjunction with ligand binding once capacitation is completed[45]. This reaction could be viewed as a series of events necessary before spermatozoa could gain access to the oocyte which is obscured by cumulus cells and a proteoglycan coat denoted the *zona pellucida.* During fertilization, spermatozoa arriving at the cumulus oocyte complex must first undergo tight binding to the *zona pellucida* leading to induction of the AR. A capacitated[46] and an intact, functional acrosome[47] is needed for penetration of the *zona pellucida* and fusion with the oocyte's plasma membrane, many of the intracellular signalling mechanisms having already been initiated during capacitation[48].

1.2.4.1 Induction by ligand receptor interaction

In vitro, the AR was initiated by the binding of the capacitated sperm's glycoprotein receptor to O-linked oligosaccharides found on *zona pellucida* protein 3 (ZP3), the spermatozoan plasma membrane as well as the inner acrosomal membrane contains receptors for *zona pellucida* proteins[49]. An example of such a receptor is P95, a 95kD phosphotyrosine membrane protein termed the *zona* receptor kinase, it was noted that the level of phosphotyrosine increases with capacitation[50].

1.2.4.2 Entry of Ca^{2+}

The AR is modulated by the selective binding and internalisation of Ca^{2+} through the outer acrosomal membrane[51]. This increase in the intracellular Ca^{2+} concentration leading to the AR was one of the earliest responses during the interaction of naturally capacitated spermatozoa with the oocyte[52].

There were thought to be three possible mechanisms responsible for modulation of the Ca^{2+} influx through the membranes of spermatozoa[53]:

- A Ca^{2+}-ATPase that could act as a Ca^{2+} extrusion pump;
- A Na^{+}/Ca^{2+} ion exchanger that had been proposed to pump Na^{+} out of the cell and Ca^{2+} into it;
- Ca^{2+} channels capable of permitting a large Ca^{2+} influx.

The most likely mechanism was the Ca^{2+}-ATPase ion exchange pump, active in the presence of Ca^{2+} at pH 9.0, since it was found that there was insufficient movement of Na^{+} for the Na^{+}/Ca^{2+} exchanger to be considered and the Ca^{2+} channels were not present in the required quantities to explain the observed Ca^{2+} influx[37]. The regulation of the Ca^{2+}-ATPase is complex as a result of the signal transduction pathway/cascade that was eventually responsible for the Ca^{2+} influx. The regulation was not by way of a directly linked sperm-agonist interaction and activation of the Ca^{2+} channels, but rather through a multi-step process involving intervening actions and intracellular pH changes[29].

1.2.4.3 Internal modifications

Changes to the outer acrosome membrane observed during the AR include swelling, crenulations, and disintegration of the 20 nm intramembrane particles' geometrical arrangement leading to fragmentation of the acrosome[54,55].

Internal modifications as a result of the Ca^{2+} influx include a rise in the pH due to the outflow of H^{+} ions, as well as intra-acrosomal protein modification of, among others, pro-acrosin to acrosin[56].

1.2.4.4 Exocytosis

The Ca^{2+} influx causes multiple fusions, vesiculation[57] and fenestrations between the outer acrosomal membrane and the inner acrosomal plasma membrane. This enables the release of the acrosomal contents, i.e. the acrosomal enzymes and associated contents pass through these fenestrations[58] coming into contact with the surface of the *zona pellucida* and the surrounding extracellular space[59]. Eventually, the entire acrosomal cap disintegrates and, when adjacent to the *zona pellucida,* this was followed by tight binding to *zona pellucida* protein 2 (ZP2) by ZP2 receptors on the sperm head previously covered by the acrosome[45].

1.2.4.5 Acrosomal enzymes

During the first stages of the AR, hyaluronidase was released[60], emerging from the acrosomal space of capacitated spermatozoa through the pores created by membrane fusion[61]. This enzyme aids the progress of spermatozoa through the hyaluronic acid matrix between the cells of the *cumulus oophorus* that surround the oocyte. Hyaluronidase was localised away from the inner acrosomal membrane and closer to the outer acrosomal membrane in the anterior acrosomal region[62].

Another enzyme released from the acrosome was acrosin. It is a 30 kilo Dalton (KD) monomer with an optimum pH of 8.5 and was viewed as a vital proteolytic enzyme similar to trypsin. This acrosomal enzyme functions to digest the *zona pellucida*[63].

At the time of ejaculation, acrosin was almost entirely present close to the inner acrosomal membrane towards the rear of the acrosome in the form of proacrosin[64], and was apparently not bound to the outer membrane. This was in contrast to hyaluronidase which was localised in the anterior region closer to the outer

acrosomal membrane and was thus released before acrosin during the AR[65]. Evidence supporting this view was that after membrane isolation, more than 70% of the acrosin activity remains associated with the inner membrane which was still attached to the sperm head[19]. This localisation results in acrosin leaving the acrosome somewhat later than hyaluronidase[66], enabling acrosin to fulfil its proteolytic function of digesting the *zona pellucida* proteins since hyaluronidase had cleared the way through the *cumulus oophorus*[19]. In the lysis of the *corona radiata* cells that obscure the *zona pellucida,* another hydrolytic acrosomal enzyme, *corona radiata*-penetrating enzyme (CPE), active at pH 7.7, was found responsible for aiding in the progression of spermatozoa past this cell mass[67].

The chief structural protein of the human body is collagen. The collagenase in the acrosome was needed to lyse this protein when encountered in the *cumulus oophorus* while neuraminic acid is a component of the *zona pellucida* layer and the enzyme most exclusively bound to the inner acrosomal membrane, neuraminidase, was believed to be responsible for digesting this acid[68].

Acrosomal lysosomal enzymes[69] include: acid phosphatase[70], β-glucoronidase, arylaminidase[71], arylsulphatase[72], β-N-acetylglucosaminidase[73], phospholipase A, non-specific esterase[74], β-aspartyl-N-acetylglucosamine-amino-hydrolase[75], and acid proteinases[76]. These enzymes all aid the digestion of proteins, lipids and carbohydrates which may obscure the *zona pellucida*.

1.2.4.6 Artificially inducing the acrosome reaction

Insight has been gained into the mechanism, role and diagnostic potential of the AR by making use of artificial AR inducers[77]. When it was found that abnormally high

frequencies of spontaneous AR were associated with unexplained IVF failure it was suggested that comparative assessment be done so that the induction of the AR could be investigated using more than one method of induction[78]. The *zona pellucida*, or more particularly ZP3, was the most suitable biological inducer for comparative assessment. In view of the restricted availibility of ZP3 due to it being a human tissue, the use of commercially available biological and biochemical agonists was necessary.

The Ca^{2+} ionophore challenge test was developed by Cummins and fellow researchers[79] after it was found that the AR must be precisely timed with respect to sperm-*zona pellucida* interaction in order for *zona pellucida* penetration to occur[80]. Inducability of the AR with Ca^{2+} ionophore A23187 was found to be of prognostic value for sperm fertilization capacity[81]. Progesterone (P) was another effective AR inducer and resulted in significant increases in AR frequencies in normozoospermic patients, but had no significant effect on spermatozoa from oligozoospermic men[82]. A significant correlation was observed between fertilization rate and P-stimulated AR frequency[83].

AR frequencies obtained for the same sample may include the measured frequency of spontaneous reacted spermatozoa, as well as the frequencies observed with P- or Ca^{2+}-induced AR. The comparative test considers these frequencies, thus enabling a more reliable measurement of acrosome reactability than would be possible if only one of the reaction rates was considered[79].

Responses varied according to morphological characteristics. A constant, non-specific response was only seen when using Ca^{2+} ionophore induction. Spontaneous

AR rate was lowered in samples exhibiting declining morphology, and the same condition saw diminished P-stimulated AR response[84].

1.2.5 Acrosomal abnormalities

Abnormalities in the structure and functionality of the acrosome may result in spermatozoa not being capable of natural fertilization, depending on the severity of the abnormality. Primary acrosomal abnormalities originate during spermatozoan development and differentiation. Such primary abnormalities include abnormal and disorientated microtubules due to reduced tubulin synthesis as a result of tumour activity. Abnormalities such as acrosomal hypo-development and even acrosomeless spermatozoa may result from missing manchette components after genetic insertion mutations[85]. Secondary acrosomal abnormalities or alterations originate from external factors such as aging or damage to the plasma and outer acrosomal membranes. A well known example was the acrosomal damage observed when examining incorrectly cryopreserved spermatozoa[86].

In summary, the acrosome plays an important role in fertilization and this was well illustrated by the following: a normal acrosome was a pre-requisite for normal sperm morphology, which seems to be a very good predictor for fertility both *in vivo*[87] and *in vitro*[88].

Research conducted by Söderlund and Lundin in 2001 on 81 patients with <5% morphologically normal spermatozoa showed that the fertilization rates were significantly lower (40%) in the group that had an acrosome index (AI) <7%. This group was compared to a second group of patients (n=70) also with <5%

morphologically normal spermatozoa but with AI ≥7%, highlighting the importance of the acrosome apart from the other factors influencing sperm morphology[89].

1.3 SPERM KINEMATICS

Spermatozoa develop the ability to swim as they pass along the epididymis. Once ejaculated, mature spermatozoa were immediately capable of the progressive movement essential for natural fertilization[21]. Research illustrates the importance of acceptable kinematics for fertilization and the value of kinematic assessment in gauging fertilization potential[90,91,92].

Kinematic parameters (Figure 3) clarify the complex movement characteristics of spermatozoa.

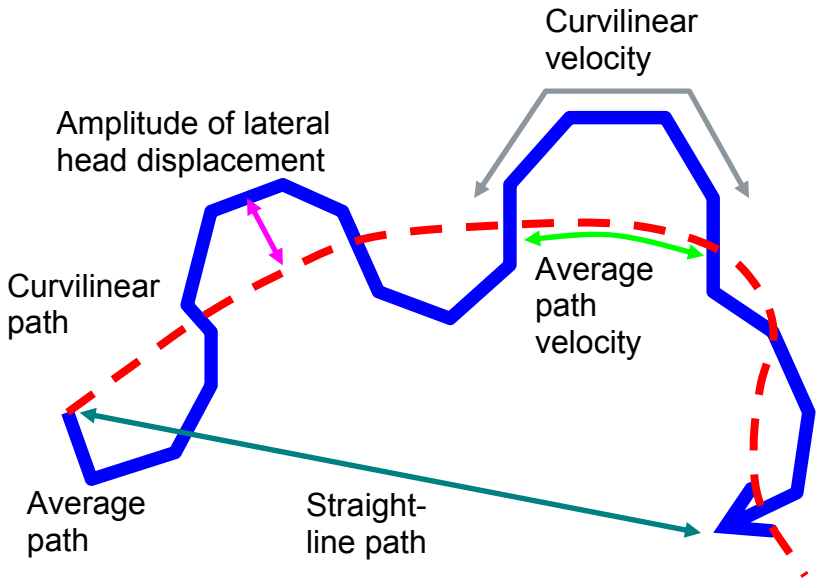


Figure 3 Different kinematic parameters of a single sperm track.

Kinematic parameters that may be quantitatively analysed by means of computer assisted semen analysis (CASA) include the following:

- Motility – the percentage of motile spermatozoa (> 50% = normal[26])

- Progressive motility – the percentage of progressively motile spermatozoa (> 25% = normal[26])
- VCL – curvilinear velocity measured in µm/s. This is the time-average velocity of a sperm head along its actual curvilinear path, as perceived in two dimensions in the microscope[26]
- VSL – straight line velocity measured in µm/s. This is the time-average velocity of a sperm head along the straight line between its first detected position and its last detected position[26]
- VAP – average path length measured in µm/s. This is the time-average velocity of a sperm head along its average path. This path is computed by smoothing the actual path according to algorithms contained within the CASA instrument's software[26]
- ALH – amplitude of lateral head displacement measured in µm. This is the magnitude of lateral displacement of a sperm head from its average path. It could be expressed as a maximum or an average of such displacements. It should be kept in mind that different CASA instruments compute ALH using different algorithms. Values were thus not strictly comparable[26]
- LIN – linearity. The linearity of a curvilinear path, VSL/VCL[26]
- STR – straightness. Linearity of the average path, VSL/VAP[26]
- BCF – beat cross frequency (beats per second). The average rate at which the sperm's curvilinear path crosses its average path[26]
- Percentage rapid cells – velocity distribution of rapid cells[26]
- Percentage medium cells – velocity distribution of medium cells[26]
- Percentage slow cells – velocity distribution of slow cells[26]
- Percentage static cells – velocity distribution of static cells[26]

Thrust from the tail was required for successful penetration of the *zona pellucida* and in order to reach the oocyte progressive spermatozoa required sufficient levels of VSL. The velocity parameters VCL, VSL, and VAP as well as the straightness of the path play a role in dictating the rate at which spermatozoa reach the cumulus oocyte complex and possibly fertilize the oocyte. VSL was found to correlate significantly with pregnancy rate [93].

Hirano *et al.* have shown significant correlations between fertilization rates and kinematic parameters such as: ALH, VCL, VSL, and the rapid movement of spermatozoa (Rapid) for samples evaluated pre-swim-up[94]. The same study showed significant correlation between fertilization rates and STR in post-swim-up samples.

It was advised that kinematic parameters were not absolute predictors of fertilization potential and that morphological characteristics, predicted by either manual or automated means, could be advantageously included in predictive models[95,96].

1.4 SPERM MORPHOLOGY AND MORPHOMETRY

The morphology of spermatozoa had long been regarded as an indicator of fertility and research had indicated that a significant correlation exists between morphology and fertilization capacity [97,98,99]. Similarly, poor morphology had been associated with deviant kinematics and inefficient penetration of both cervical mucous and the *zona pellucida*[100,101].

The first classification for human spermatozoa was introduced by MacLeod[102], making use of six different categories to allow for the variation in shape and size of

spermatozoa. In 1966, Freund also published a classification system with six classes,.His system addressed the sperm head, as well as tail defects and immature spermatozoa[103].

A new approach of taking all observed defects into account was proposed by Eliasson in 1971[104]. He counted all defects (of the head neck, midpiece and tail) separately and expressed this as a fraction of the number of cells analysed. Eliasson was the first to assess the actual morphometrical properties of cells, enabling him to reject defective cells on the basis of size. In 1975, David developed an elaborate morphological evaluation system making use of an eventual average number of abnormalities per individual. All evaluated abnormalities were considered to be of equal relevance and thus contributed equally towards the average[105].

In 1980, the first WHO classification in the form of the "WHO laboratory manual for the examination of human semen and semen-cervical mucus interaction" was published (WHO 1980) [106]. Subsequent revised editions were updated to include more strictly defined parameters and the manual became the standard in the examination and classification of seminal characteristics[25,107]. Concurrently, the Düsseldorf classification was developed as a system of classification that laid more emphasis on the acrosomal defects and the elongation of post-acrosomal regions of the spermatozoa.

Building on the foundation laid by Eliasson *et al*.[105], the role of normal sperm morphology was explored further and research resulted in additional morphological classifications being developed, amongst others, the Tygerberg strict criteria[108]. This classification system was based on the morphology of spermatozoa found in the

internal cervix after clear and decisive selection[109] by the cervical mucus during natural migration of the spermatozoa into the uterus for a population showing a more uniform morphological appearance[110]. The term “strict” refers to the method of classifying all borderline or slightly abnormal morphological forms to be abnormal. The use of the Tygerberg strict morphological assessment criteria has also been described as a prognostic indicator for fertilization rates in both assisted reproduction and *in vivo* fertilisation and conception[109]. According to the Tygerberg strict criteria significantly lower fertilization rates were observed in individuals having < 14% normal morphology[111]. It was also found that, in comparison with previously used classifications, much better inter- and intra-observer correlation was achieved using the Tygerberg strict morphological assessment criteria[112]. The incorporation of the Tygerberg strict morphological assessment criteria in computerised analyses[113] was seen in software such as the Metrix morphometrical analysis program employed by the Hamilton-Thorne integrated visual optical system (IVOS).

For spermatozoa to be classified as having normal morphology according to WHO (1999) criteria, the sperm head, neck, midpiece and tail must all be normal. The head should show an oval acorn shape with a length of between 4.0 and 5.0 μm while the width must be between 2.5 and 3.5 μm. The elongation may not exceed a length-to-width ratio of less than 1.5 or more than 1.75. These ranges were defined for Papanicolaou-stained cells and were also used when rapid staining methods such as the Hemacolor and Diff-Quik stains were employed. The acrosomal region should comprise 40-70% of the head area and be well defined. In addition, the midpiece should be slender, less than 1 μm in width and make up one and a half times the length of the head and attached axially to the head. Cytoplasmic droplets may not exceed 49% of the head size and the tail should be about 45 μm long, uncoiled and

thinner than the midpiece[108]. Menkveld *et al.*[114] found support for this definition of morphological normality by examining the morphological appearance of spermatozoa tightly bound to the human *zona pellucida* as observed in the hemi*zona* assay and during *in vitro* fertilization[115].

Sperm morphology had been shown to be one of the best indicators of fertilization potential[116,88]. Lack of a single world standard for the analysis of morphology and the estimation of parameters was still problematic and was observed to cause reduced accuracy and reliability[98,117] This observed inaccuracy and reduced reliability could be overcome by strict quality control. It was shown that when adequate quality control procedures were included in the design they enabled the use of manual evaluation and analysis of morphological parameters as prognostic factors[118].

The use of automated systems was one attempt at reducing analysis variation and obtaining standardized results. Ombelet found that more precise determination of morphological classification and fertilization potential could be achieved by reducing analysis variation. This would enable andrologists to accurately determine fertility status and identify suitable treatment options in cases where subfertility was identified[119].

Sperm morphometry refers to the quantification of the physical dimensions of structures forming part of spermatozoa. Quantitative measurement of these dimensions may be performed with the aid of computerized automated sperm morphology analysis (ASMA) systems. The hardware consists of a microscope, a video camera, a computer, a frame grabber and the morphometrical software used to evaluate the images captured. It performs quantitative analysis and provides

statistically useful data. The value of these systems lies in the ability to perform repeatable and automated analyses, more swiftly than when done manually, based on set parameters. These systems were intended to rapidly provide objective, quantitative morphometrical data[96]. Irvine *et al.*[120] confirmed that morphometrical data obtained by means of ASMA using a Hamilton-Thorne IVOS system was significantly related to time to conception and that CASA could be reliably used in routine andrology [97]. In 1999, Krause *et al.* offered supporting views as they reported superior performance of computerised systems for the dimensions of structure and process in their study[121]. In the same year, however, the fourth edition of the WHO manual for the examination of human semen was published and noted that "several studies have suggested that assessment of sperm morphology using computerized methods may provide clinically useful information, further development was however needed before computer-aided sperm (morphology) analysis could be recommended for routine assessment of sperm morphology" [122].

1.5 MICROSCOPIC CASA AND ASMA ANALYSIS

CASA systems were used to evaluate kinematic parameters, while ASMA systems were utilized to determine the morphometry of spermatozoa[123]. The use of automated image analysers was an attempt to address the problem of decreased reproducibility due to observer variation. A decrease in the analysis variation of a sample or patient may be observed since automated recognition of spermatozoa depends on reproducible software responses based on preset threshold settings for shape, size, intensity, morphometrical parameters, kinematic patterns, etc.[124]. Several parameters (such as morphology and VSL) of semen have been shown to be significantly related to conception *in vivo*. Included in these were kinematic parameters, sperm concentration, tail properties and morphological aspects[93,94].

1.5.1 Computer-assisted semen analysis of kinematic parameters

CASA provides the andrologist with kinematic data that show good repeatability and reliability between laboratories and technicians and makes the acquisition of precise quantitative data possible[124].

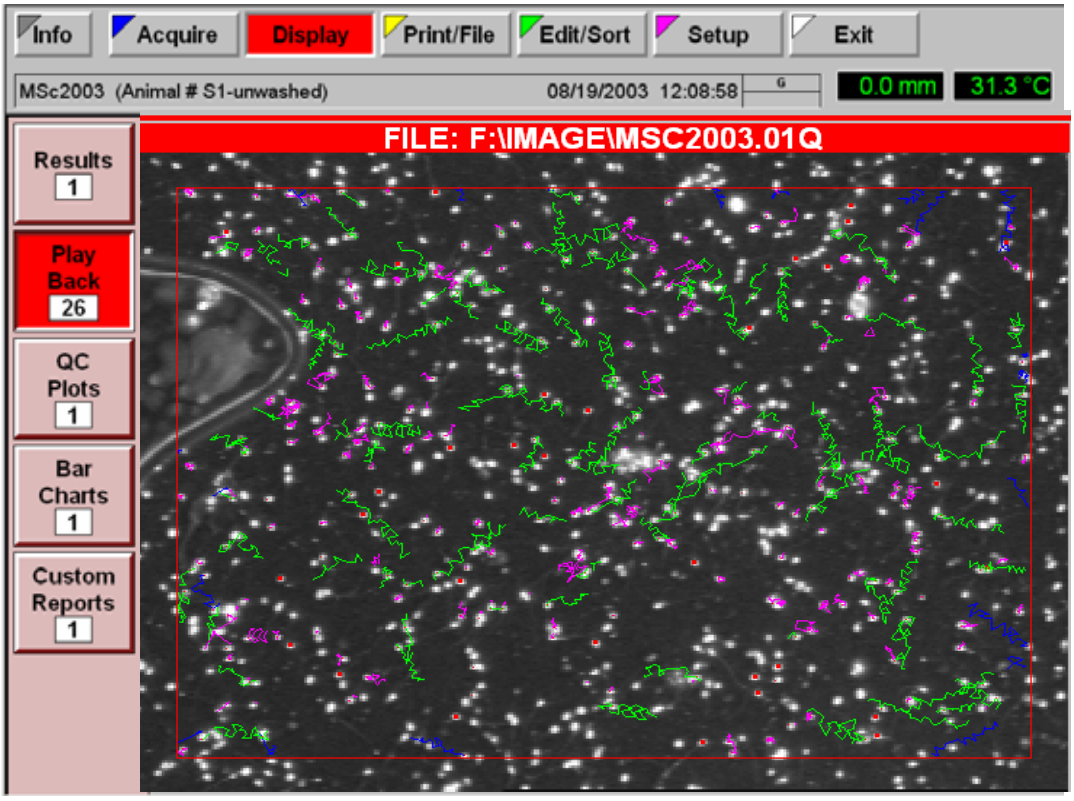


Figure 4 CASA may be used to analyse the motility and other kinematic parameters of spermatozoa.

The ability of CASA systems to determine and generate objective kinematic measurements of motile spermatozoa populations may be applied to gauge the fertilization potential of the population, and thus be used to formulate differential diagnoses. Kinematic data obtained using CASA systems have previously been shown to be predictive for both *in vivo* fertilization and IVF[125].

An example of an image captured using a CASA system (Hamilton Thorne IVOS) is displayed in Figure 4. The green lines represent progressively motile spermatozoa, the pink represents non-progressive but motile spermatozoa, the red dots represent immotile cells and the blue lines trace spermatozoa that moved outside of the observation area during the analysis

As with the introduction of any new technologies and instruments, the suggestion of using CASA for routine automated assessment of human semen in andrology laboratories was greeted with scepticism. Barratt was the first to publish his findings on the predictive value of CASA. His work demonstrated that semen concentration and sperm motility as well as the percentage of progressively motile cells, were all significantly related to the time to conception[126]. Further work has highlighted the predictive value of individual kinematic parameters with respect to fertilization potential[94].

1.5.2 Automated sperm morphometry analysis

For ASMA systems, designed to quantitatively analyze the morphometry of fixed and stained spermatozoa, standardized slide preparation and staining was required since the systems were designed to utilize threshold values for parameters such as size and colour intensity to differentiate and detect individual spermatozoa. Correct preparation may thus aid the automated systems to achieve greater precision, repeatability and validity in measurement, evaluation and recognition of spermatozoa[127]. To achieve this consistency, the standardized protocol for slide preparation must be rigorously followed.

In cases where standardized protocols were followed, excellent agreement and minimal variance were observed with respect to repeated analysis of the same spermatozoa[128]. There were, however, mixed reports of variance between slides made from the same semen sample, the results ranging from less than one percent variance to significant variance, possibly due to the innate inconsistency of semen samples[129,130]. In addition, it had been shown that there was excellent correlation between different instruments of the same make and model with respect to analysis results of the same samples[131].

It was found that percentage spermatozoa with normal acrosomes (excluding all other abnormalities), expressed as an acrosomal index, had specific advantages in comparison to sperm morphology assessment in the prediction of *in vitro* fertilization outcome[132]. This trend was more pronounced in a study group that obtained a “poor prognosis” i.e. < 4% normal morphology observed[133]. There was conflicting use of the term “acrosome index”. The term had been used to describe the size of the acrosome, however, current literature and this work used the term acrosome index (AI) to represent the percentage spermatozoa in a population with normal acrosomes, irrespective of other spermatozoa head abnormalities[133]. Morphological and acrosomal differences observed with the aid of ASMA could provide an explanation as to why some patients, with a very low morphology score, still have a reasonable fertilization rate during *in vitro* fertilization and why others do not[134].

Since the automated evaluation of spermatozoa parameters was influenced by a large number of factors, the assessment of individual parameters, although helpful, would be insufficient[135]. Accurate, simple and standardised assessment of multiple parameters, made possible by the use of automated systems that compare

favourably with manually obtained parameters, was most advantageous[124]. Such data may aid in the understanding of *in vivo* processes and be used to determine suitable treatments for patients to be treated using assisted reproductive techniques.

Figure 7 depicts an individual spermatozoon and some of the parameters that may be analysed using an ASMA system's analysis software (Hamilton Thorne). This includes the measurements of the head length and width, elongation, head area, circumference and the acrosome percentage. From these measurements, other data such as the acrosome size (AS) may be calculated.

Automated morphological evaluation using very strict criteria had been found not only to predict fertilization rate *in vitro,* but also the rate of conception in individual couples accepted in an *in vitro* fertilization program[136]. Ombelet, however, found that sperm morphology only becomes a very useful predictive tool in a subgroup of patients that display severe subfertility[137].

1.5.3 Limitations of automated systems

Accuracy of ASMA systems, as is the case with manual evaluation, relies on procedural aspects of analysis such as the preparation and staining of slides and smears, as well as the materials, such as the quality of optics and magnification of the light microscope used[124].

It had been shown that although the sensitivity of CASA for the prediction of fertilization was high, the diagnostic specificity was low[138]. What this means is that at present, kinematic data obtained using CASA is better at predicting when fertilization would be successful than at predicting when fertilization would fail.

In addition, it was found by Davis and Katz that technical problems such as inaccuracy of count and percent motility for low and high concentration specimens, confusion over the presence of debris and different implementations of algorithms across instruments still persist[139].

1.6 BINDING OF SPERMATOZOA TO *ZONA PELLUCIDA* PROTEINS

Spermatozoa need to bind to the *zona pellucida* in order to direct their thrust through the *zona pellucida* and penetrate the oocyte[45]. The initial tight binding of spermatozoa to the *zona pellucida*, or more specifically to ZP3, was regarded as a crucial and necessary step to *zona pellucida* penetration and subsequent fertilization. The spermatozoa binding to ZP3 was followed by tight binding of receptors located on the inner acrosomal membrane to ZP2. Subsequent penetration allows entry of spermatozoa into the perivitelline space[140,141].

The two most common tests to evaluate this binding and subsequent penetration were the hemi*zona* assay[142] and the competitive intact *zona* binding assay[143]. It is worth noting that both these tests incorporate the assessment of tightly bound spermatozoa as their endpoint and both have been demonstrated to have a high predictive value for fertilization results under *in vitro* conditions[144].

Morphology had been shown to be the best predictor regarding the ability of spermatozoa to bind to the *zona pellucida* under assay conditions[145]. It had further been established that the hemi*zona* assay had a particularly virtuous capability to identify male factor cases at risk of failing fertilization[146] and in theory capable of predicting male infertility[147]. The hemi*zona* assay provides an ideal functional and

homologous model to simultaneously investigate multiple events required for successful fertilization. Thus, the multiple sperm functions necessary for successful fertilization were closely associated with the ability of the spermatozoa to undergo tight binding of the hemi*zona*'s *zona pellucida*[148].

1.7 SELECTION OF SPERMATOZOA FOR INFERTILITY TREATMENT

Separation of spermatozoa from the seminal fluid as well as natural selection of subgroups of the ejaculated population occurs when spermatozoa progress through the cervical mucus. In assisted reproductive procedures such as routine *in vitro* fertilization (IVF) and intra-uterine insemination (IUI), this separation is performed in the laboratory[149]. This separation protects the spermatozoa from extended exposure to the seminal fluid and increases the proportion of motile and morphologically normal spermatozoa in the fraction to be used during treatment[150]. The change seen in kinematic parameters of the selected population compared with the ejaculated population indicates the type of selection that takes place during the swim-up preparation of samples to be used for assisted reproductive techniques such as *in vitro* fertilization (IVF)[151] and artificial insemination[152].

1.8 MOTIVATION AND OBJECTIVES

From the literature it was clear that sperm kinematics as well as both morphology and morphometry were associated with fertilization potential. Variances in these characteristics could contribute to higher than expected fertilization success or conversely unexplained fertilization failure. It was also evident from the literature that

no clear consensus exists regarding the relationships that exist between sperm kinematics, morphometry, morphology, and sperm *zona* interaction.

The objective of this study was thus to evaluate the possible relationship between morphometrical characteristics, and kinematic characteristics in human spermatozoa. In order to achieve this objective CASA was employed to determine kinematic parameters and ASMA was used to analyse morphometrical measurements in pre- and post-swim-up human sperm populations.

The results are also to be statistically compared in order to determine whether any correlations exist between kinematic and morphometrical parameters, especially thos related to acrosomal characteristics.

CHAPTER 2
MATERIALS AND METHODS

The experimental protocol as performed by the candidate including the materials used and the subsequent methods applied to obtain the different measurements, will be discussed in detail in this chapter. The following objectives were set for the evaluation of the 30 samples collected from 7 donors:

- Investigate pre- and post-swim-up kinematic parameters.
- Evaluate pre- and post-swim-up morphometrical parameters with the use of computerised measurements.

2.1 ANALYTICAL PROTOCOL

This study was designed as a prospective analytical study making use of randomly selected sperm donors. This study was approved by the Institutional Review Board's ethical committee. The step by step experimental protocol in Figure 5 depicts the sequence of analyses. Directly after liquefaction, samples were macroscopically analysed and microscopically evaluated for both kinematic and morphometric characteristics. Samples were washed and a swim-up selection performed after which the sample population was again microscopically evaluated for both kinematics and morphometrics.

2.2 SEMEN PREPARATION

Semen samples (n=30) were obtained from seven randomly selected healthy donors between the ages of 22 and 35. Subjects were asked to abstain from sexual activity for 2-3 days before the samples were collected by masturbation into a sterile plastic

specimen container[108]. The ejaculate was incubated at 37 °C for 30 minutes to allow for complete liquefaction to take place. Semen parameters were measured immediately after liquefaction, after which the sample was divided into the different aliquots for the various experiments.

2.2.1 Washing of sample

Washing of semen enables removal of acellular constituents such as prostaglandins, infectious agents and antigenic proteins[120]. Washing and resuspension also allows for adjustment of the concentration of a semen sample as needed for computerised evaluation. Samples that underwent washing were treated in the following manner:

1) Add Ham's F-10 medium to the sample and make up to 5 ml;
2) Centrifuge at 1800 revolutions per minute (rpm) (400 g) for five minutes;
3) Aspirate supernatant from pellet;
4) Add Ham's F-10 medium to the pellet and make up to 5 ml;
5) Resuspend pellet by shaking by hand;
6) Centrifuge at 1800 rpm (400 g) for five minutes;
7) Aspirate supernatant from pellet leaving only the pellet and a minimal amount of media, being careful not to disturb the pellet.

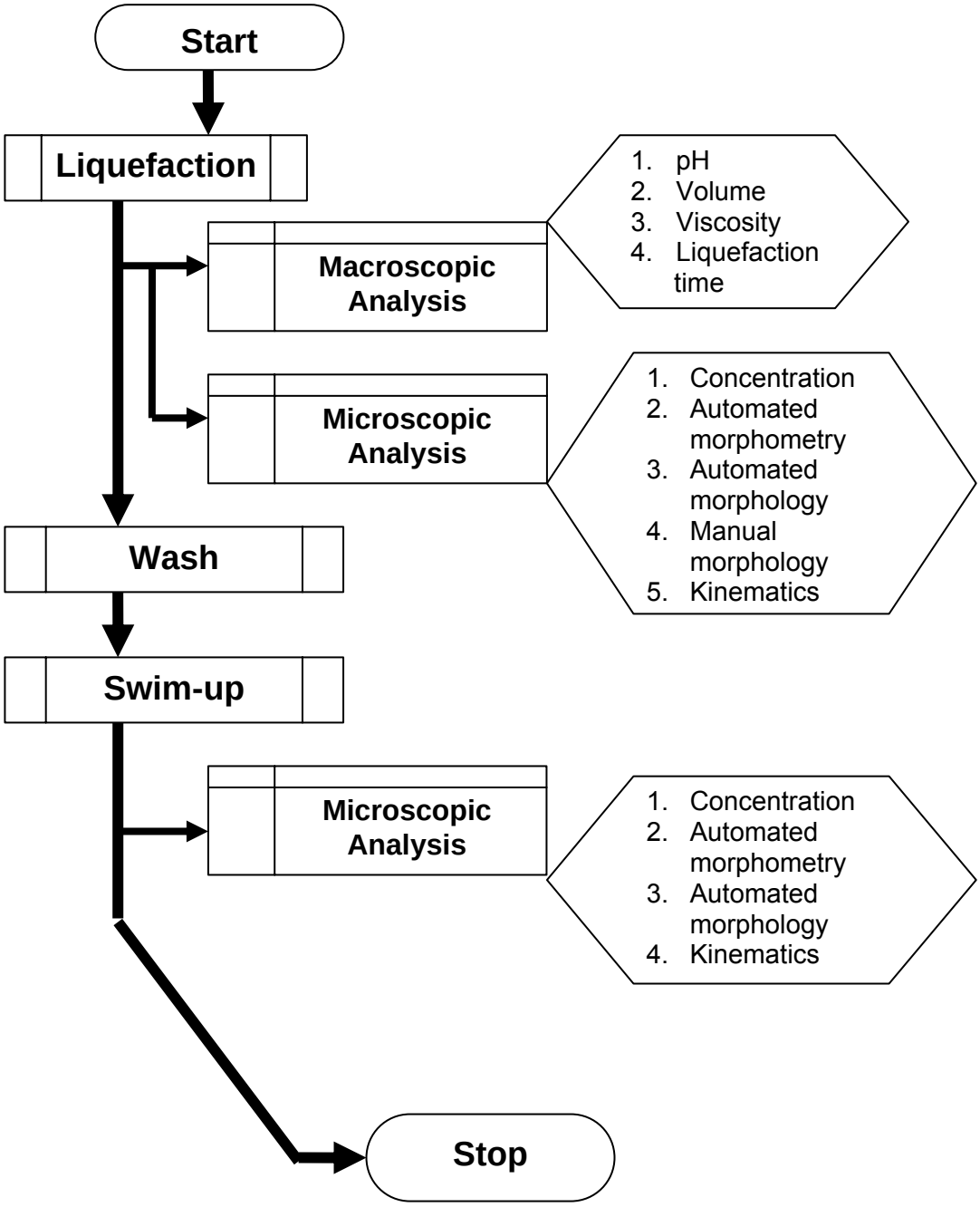


Figure 5 Flow chart outlining the sequential experimental protocol followed during this study.

2.2.2 Swim-up

Swim-up was carried out after washing. The separation of motile spermatozoa from immotile spermatozoa and debris occurs as a result of motile spermatozoa swimming from the pellet into the media layered over it. This

fraction of sperm was studied since it was the fraction used in the treatment of male factor infertility in procedures such as ICSI, IVF, GIFT, SUZI and PZD[153].

1) 1ml of Ham's F-10 containing BSA (3%) was carefully added to the washed pellet;
2) The pellet was resuspend by shaking or running the Eppendorf tube along the top of a test tube rack, do not vortex;
3) Centrifuge at 1800 rpm (400 g) for five minutes;
4) Place in an incubator at an angle of 45° and at 37 ºC and 5% CO_2 for 30 minutes;
5) Upon completion of swim-up, the top 300-400 µl was aspirated for further experimentation.

2.3 CONCENTRATION ADJUSTMENT

1) The sample was now split into two fractions that would be used in the following experimental procedures:
   a) Kinematic analysis and concentration determination;
   b) Morphometrical analysis - concentration adjusted to 100 x 10^6 cells/ml;

2.4 ANALYSIS OF KINEMATIC PARAMETERS

Kinematic characteristics of spermatozoa were analysed using CASA (Hamilton Thorne Research IVOS system, Hamilton Thorne, Los Angeles) that utilizes an internal optical system. Illumination was by means of a lighting system that utilizes a 1000 Hz strobe lamp to eliminate blurring and produce precisely tracked motion paths. The optical unit consists of a built-in Nikon microscope with an effective

magnification of 1000x, the strobe lamp, an ultra-violet lamp, a Sony XC-75 CCD video camera and an electronically controlled mechanical stage to hold the slide.

2.4.1 CASA Settings

Standard set-up parameters were used with standard dual sided cell chambers of 20 µm depth. The analyser settings were: 30 frames/60 Hz; minimum contrast, 80; minimum cell size, 3; minimum static contrast, 30; low average path velocity (VAP) cut-off, 10 µm/s; low VSL cut-off, 10 µm/s; head size, non-motile, 3; head intensity, nonmotile, 160; static head size, 1.01-2.91; static head intensity, 0.60-1.40; slow cells not motile; magnification = 2.01; and temperature, 37 °C.

2.4.2 CASA analysis technique

1) A 5µl drop of the semen sample was placed at the entrance of the chamber slide already covered with a coverslip;
2) The sample was then loaded into the analysis stage by pressing “load”;
3) The flow of the sample into the chamber was allowed to subside so as to avoid miscalculation of kinematic parameters;
4) The “info” tab was clicked on and general information such as study number, volume, dilution and subject ID# was entered.
5) The “Acquire” tab was clicked to visualize the currently viewed area of the chamber in the analysis stage;
6) The image was focused and then analysed;
7) Multiple fields were analysed in an attempt to analyse a minimum of 200 spermatozoa;
8) Fields were ignored if there was a large amount of debris or other material that could cause inaccurate measurements;

9) The results were recorded;
10) Samples with very high spermatozoa concentrations were diluted 1:1 with Ham's F10 medium for accurate determination of count by the CASA (IVOS) system.
11) In this manner the parameters; motility, progressive motility, VAP, VSL, VCL, ALH, and BCF were determined

2.5 ANALYSIS OF MORPHOMETRICAL PARAMETERS

Morphometrical data such as size, colour intensity and shape were used to classify spermatozoa. Using the integrated visual optical system (IVOS), it was possible to effectively determine the dimentions of many components which comprise the spermatozoan cell. Included in the parameters measured was the mean AS of a particular semen sample. This quantitative measurement in which the area of the acrosome was determined in μm^2 enables investigation into the effects of AS. The IVOS system was unique in that, in addition to using morphometrical measurements, it used a signature method to evaluate the shape of cells identified as spermatozoa and this method was found to have clinical significance [113].

Morphology software (Metrix Morphology v12.1, Hamilton Thorne PTY Ltd, New York, USA) was used to determine the morphometrical parameters of individual spermatozoa and thus assess the population from which they originate. Evaluations were performed with the use of 662 nm wavelength illumination in conjunction with a 100x oil-immersion objective. Sperm cells were evaluated (blindly) and the percentage of normal sperm, as calculated by the computer, recorded.

For this study, thin, evenly spread smears of fresh semen, as well as smears of the post-swim-up cells were made as per the instructions of the Metrix morphology software manual. The smears were then stained using the Hemacolor kit (Merck, Darmstadt, Germany, Catalogue No. 11661). This kit was Merck's equivalent of the DiffQuik 3186 kit produced by Baxter DADE AG. Soler *et al.*, however, found that Hemacolor staining rendered more digitized cells than DiffQuik or Papanicolaou staining for precise morphological analysis[131]. Metrix Morphology® was a dimension specific software package that was set up to correctly evaluate DiffQuik stains taking into account the effects of the stain on morphometrical parameters.

In this study, spermatozoa were considered normal when the head had a smooth oval configuration, with a well defined acrosome involving 40–70% of the head, with no visible tail, neck or mid-piece defects, and no large (> 49% of head size) cytoplasmic droplets[154]. Normal morphometrical ranges that were used for the Hemacolor-stained spermatozoa fell between the following limits: the length of the head was 4.5-5.5 μm and width of head was 2.5-3.5 μm, elongation was between 45% and 78%, head area between 8.8 μm^2 and 15.0 μm^2 and head perimeter between 10 μm and 14 μm. Hemacolor causes less swelling than Diff-Quik (normal head length 5–6 μm[86]) but more than when using Papanicolaou (normal head length 4–5 μm[86]). The normal head length for Hemacolor was 4.5-5.5 μm[131,155].

2.5.1 ASMA smear preparation

1) A 10μl drop of the sample was placed on a pre-cleaned microscope slide near the frosted end;
2) By using another slide, held obliquely to the first at a 30º angle, the slide was lowered onto the drop;

3) Once the droplet had spread along the junction of the slides, the slide was firmly pulled away from the frosted end along the length of the smear slide;
4) Place the prepared smear on the slide warmer and allow to air dry for a minimum of 15 minutes.

2.5.2 Staining procedure

1) The dried smear slide was dipped in the Hemacolor fixative for 10 seconds;
2) The edges of the slide were blotted dry with a paper towel;
3) The sperm was then stained by immersion in the Hemacolor staining solution (Solution #1) for 22 seconds;
4) The edges of the slide were again blotted with a paper towel;
5) The sperm was then counterstained by immersion in the Hemacolor counterstaining solution (Solution #2) for 24 seconds;
6) The edges of the slide were blotted with a paper towel;
7) Rinse the slide gently in distilled water;
8) The edges of the slide were blotted with a paper towel after which the slide was placed in a drying oven at 60°C and allowed to air dry fully.

2.5.3 Mounting procedure

1) Place 4 drops of mounting medium along the centre of the slide;
2) Carefully place a clean coverslip on the slide by lowering first the one side and then the other, thereby ensuring that air was not trapped between the sample and the coverslip;
3) Press the coverslip gently to distribute the mounting medium;
4) Allow mounting medium to dry sufficiently before analyzing.

2.5.4 Morphometrical analysis

Automated analysis of morphological parameters was performed on spermatozoa mounted and stained as described above and obtained from the fresh unwashed sample as well as the swim-up fraction in the following manner:

1) The mounted slide was inserted into the retractable stage of the IVOS and loaded;
2) Starting with the lowest power objective the image was brought into focus after which an objective with a higher magnification may be selected;
3) A long, large drop of immersion oil was then placed on the surface of the coverslip and the 100X objective was brought into position;
4) The sample data were entered;
5) The sample was scanned and analyzed (Figure 6)
    a) Measurements performed include: Length, width, area, elongation, circumference and acrosome percentage;
6) A review was done to correct any incorrectly analysed images;

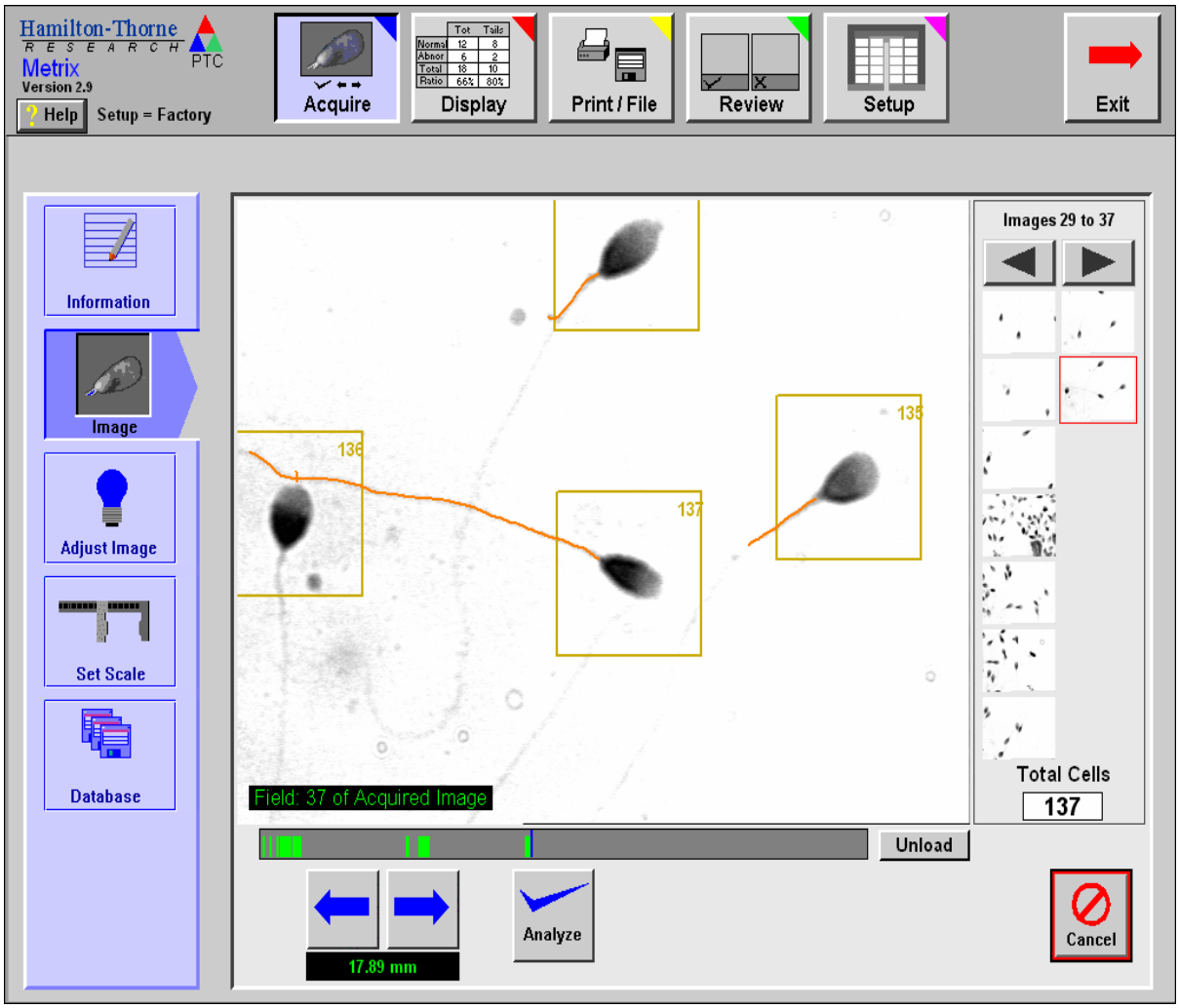


Figure 6 Analysis of a microscopic slide field using ASMA software.

7) In order to take into account both morphologically normal and abnormal spermatozoa the results were used to calculate the average AS of the morphologically normal and abnormal spermatozoa in the sample, as determined by the IVOS. Once the AS of the normal and abnormal populations was known the mean AS and acrosome index (AI) of the sample could be calculated:

   a) AS was calculated by multiplying the average head area with the acrosomal percentage;

b) AI was calculated by counting all the spermatozoa with normal acrosomes, irrespective of other abnormalities, and taking this as a percentage of the analysed population;

8) The results were recorded.

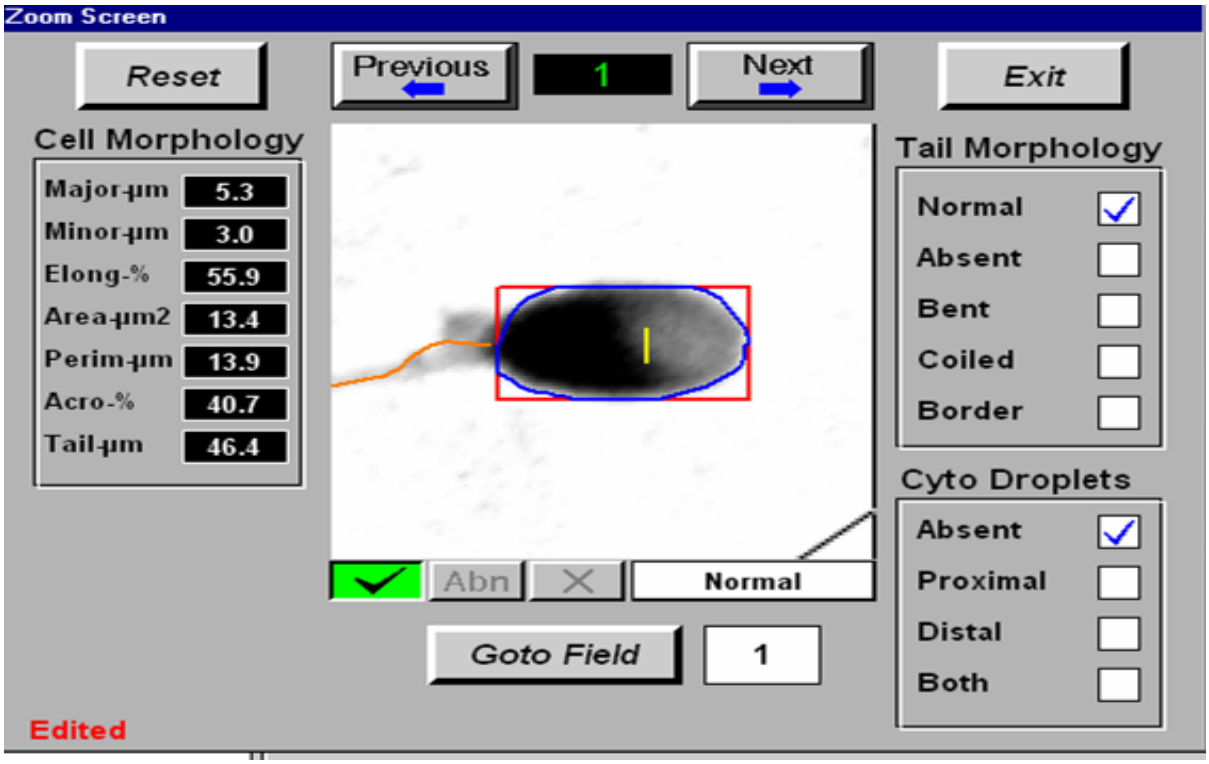


Figure 7 Parameters of individual spermatozoa may be analysed with ASMA hardware and software.

2.6 MANUAL MORPHOLOGY ANALYSIS

Manual morphology analysis of the fresh unwashed spermatozoa mounted and stained as described above was performed according to the Tygerberg Strict Criteria. Spermatozoa being regarded as morphologically normal must possess of a smooth oval head, its acrosome covering 40-70% of the head area and have a head length of 3-5 µm and a head width of 2-3 µm. The width to length ratio must be between 0.60-0.67, while the tail must be uniform and uncoiled with a length of about 45 µm[124].

Slides prepared for morphological analysis were manually evaluated under light microscopy. A minimum of 50 cells were evaluated per slide.

2.7 STATISTICAL ANALYSES AND DISTRIBUTION

All statistical evaluations and tests were carried out using GraphPad Prism 2.01. Normality of data sets was determined by the KS distance according to the Kolmogorov-Smirnov test. All measured variables with the exception of sample concentration post-swim-up were found to show normal distribution. As a result sample concentration was analysed using Wilcoxon's matched pair's non-parametric *t*-test (a modified Mann Whitney non-parametric *t*-test).

Data were expressed as mean ± standard error (SE). Student's *t*-test for paired data was used to compare the results of all the acrosome and kinematic parameter studies, while Pearson's test was used to perform correlation tests. The median and mean of the analysed and discussed data was not found to be significantly different and thus means were used and not medians. P-values equal or less than 0.05 were considered statistically significant.

CHAPTER 3
RESULTS

3.1 INTRODUCTION

The different results obtained are presented in this chapter. Data from statistical analyses focused on the relation of AS to kinematic parameters. Thirty samples were collected from 7 donors and these were evaluated for kinematic and morphometric parameters. The inclusion criteria for the data sets were:

⇒ Data sets must contain at least two post-swim-up data sets on AS.

- If, for example, data set 45 contained kinematic data for the pre- and post-swim-up population but included no measurement for AS, the data set was excluded from the statistical analysis.

⇒ A minimum of 20 cells had to be analysed by means of ASMA to determine the post-swim-up AS.

3.2 BASIC SEMEN ANALYSIS

Initially, a basic semen analysis of the 7 donors was performed and the results are displayed in Table 1 . The average volume of the ejaculate was 2.9 ml ± 1.14 with an average pH of 7.5 ± 0.35. The highest volume was observed for Donor 2 (4.8 ml) while only 1.3 ml was recorded for Donor 7. For concentration, however, Donor 7 was seen to have the second highest concentration (113 x 10^6 / ml), while Donor 4 was seen to have the highest concentration (251 x 10^6 / ml), more than eight times the recorded volue for Donor 3 (29.4 x 10^6 / ml). All the pH values remained within normal limits (7.5 ± 0.5) and the average motility of 40.1% was almost double that of Donor 5 (22%). Donors 4 and 7 were seen to have the highest and second highest motility, as was the case with the concentration. Donor 4's sample had an average motility of 56% while Donor 7's sample had an average motility of 55%. Manual morphology results are discussed under morphometric measurements (page 60).

Table 1 Initial examination of 7 donors gave the following pH, macroscopic and microscopic results.

Subject ID	Vol (ml)	pH	Concentration (million/ml)	Motility (%)	Morphology (% normal)
1	3.6	7.9	46.3	27	8
2	4.8	7.8	70.9	39	10
3	2.6	8.0	29.4	41	9
4	1.9	7.4	251.1	56	12
5	3.0	7.3	41.3	22	10
6	3.2	7.3	72.4	41	12
7	1.3	7.1	113.0	55	9

On average the ejaculates contained 89.2 x 10^6 cells/ml with an average motility of 40.1% and mean morphology, according to the Tygerberg Strict criteria, of 10.3 ± 1.53% normal spermatozoa.

3.3 MICROSCOPIC ANALYSIS

Several different microscopic analyses were performed on the semen fractions, these included kinematic and morphometric analysis which were performed on both pre- and post-swim-up populations of spermatozoa. Both after liquefaction and as soon as swim-up had been completed, kinematic analyses were performed on the pre- and post-swim-up samples. Concurrently, smears were made from post-swim-up populations of spermatozoa to be used in the morphometric analyses.

3.3.1 Concentration

The average pre- and post-swim-up concentration for semen samples of each individual was determined by means of CASA. As is seen in Table 2, the mean pre-swim-up concentration was 72.72 x 10^6 /ml, while the post-swim-up concentration

averaged only 3.65 x 10^6 /ml. In all the subsequent tables, measurements were tabulated as averages ± SE for each individual.

Table 2 The average pre- and post-swim-up sample concentration of 7 donors.

Subject ID	Concentration (million/ml)							
	n	Pre-swim-up			n	Post-swim-up		
1	3	34.80	±	6.50	3	1.73	±	0.47
2	6	65.85	±	10.62	6	4.18	±	0.46
3	5	145.70	±	11.40	5	5.62	±	1.32
4	3	34.23	±	10.78	3	3.10	±	1.27
5	3	65.53	±	14.00	3	5.33	±	1.08
6	5	47.20	±	12.87	5	2.60	±	0.68
7	5	115.70	±	21.16	5	2.98	±	0.32
Average	**30**	**72.72**	**±**	**16.07**	**30**	**3.65**	**±**	**0.55**

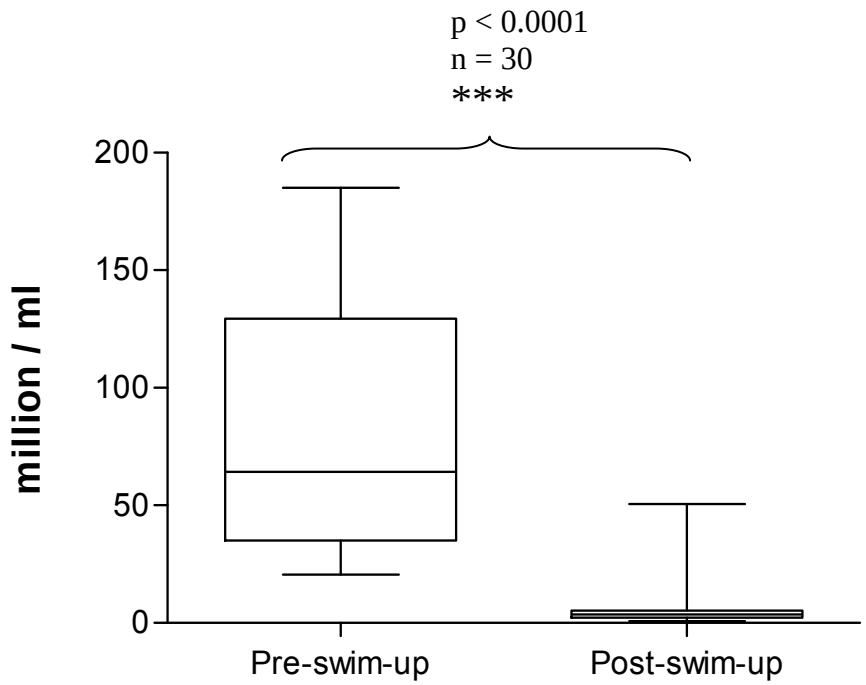


Figure 8 The average pre-swim-up and post-swim-up spermatozoan concentrations of all the samples.

As is seen in Table 2, before swim-up the average sample concentration of all 7 donors was determined to be greater than 20 million/ml thus exceeding the minimum reference value of 20 million/ml recommended in the 1999 WHO manual[26]. Donor 3

averaged a pre-swim-up concentration greater than 140 million/ml while Donors 1 and 4 averaged sample concentrations below 35 million/ml.

Of the examined post-swim-up populations Donor 1 displayed the lowest concentration, this time 1.73 million/ml while the other donors vary between 2.6 million/ml and 5.6 million/ml. We thus observe Donor 3's average concentration to consistently be the highest in this group both pre- and post-swim-up while Donor 1 shows the second lowest concentration in pre-swim-up and lowest concentration post-swim-up results. This difference in concentration was thought to be chiefly due to the large number of spermatozoa that remain in the pellet after centrifugation.

The degree of difference in sample concentration pre- and post-swim-up is depicted in Figure 8 by means of a box and whisker plot. A significant difference ($p < 0.0001$) was found between sample concentration pre- and post-swim-up selection.

3.3.2 Kinematics

Kinematic parameters were determined pre- and post-swim-up for the semen samples of each individual. The various parameters measured by means of an automated system will subsequently be discussed.

Motility:

As recorded in Table 3, the mean pre-swim-up motility for the seven donors was 38.9 ± 2.64%, post-swim-up this increased to 75.4 ± 2.96%. Pre-swim-up, Donor 1 showed the lowest percentage of mobile spermatozoa (30.0 ± 4.04%). Post-swim-up this increased to reflect the second highest average motility within the group (81.0 ± 5.20%). Donor 5 was reported to have the lowest mean motility and highest

post-swim-up SE (64.3 ± 15.19%), after having recorded the second lowest motility and highest sample SE pre-swim-up (30.67 ± 10.8%). Pre-swim-up the highest mean motility was observed in Donor 3's samples (48.2 ± 3.28%), while it further significantly improved post-swim-up. The average motility differed significantly between the pre- and post-swim-up populations ($p < 0.0001$, Figure 9).

Table 3 The percentage motile and progressively motile spermatozoa of donors' samples pre- and post-swim-up were evaluated.

Subject ID	Motility (%)								Progressive Motility (%)							
	n	Pre-swim-up			n	Post-swim-up			n	Pre-swim-up			n	Post-swim-up		
1	3	30.00	±	4.04	3	81.00	±	5.20	3	13.00	±	3.61	3	32.33	±	10.27
2	6	42.67	±	4.26	6	76.00	±	9.19	6	11.83	±	3.39	6	27.17	±	5.64
3	5	48.20	±	3.28	5	73.40	±	10.22	5	12.80	±	2.48	5	35.20	±	9.24
4	3	36.67	±	4.18	3	89.00	±	3.06	3	13.33	±	5.04	3	10.67	±	0.67
5	3	30.67	±	10.84	3	64.33	±	15.19	3	11.67	±	4.63	3	19.67	±	18.19
6	5	38.80	±	4.68	5	71.00	±	7.85	5	15.20	±	1.69	5	16.40	±	3.85
7	5	45.20	±	5.72	5	73.20	±	4.47	5	16.60	±	4.34	5	19.80	±	5.57
Average	**30**	**38.89**	**±**	**2.64**	**30**	**75.42**	**±**	**2.96**	**30**	**13.49**	**±**	**0.68**	**30**	**23.03**	**±**	**3.35**

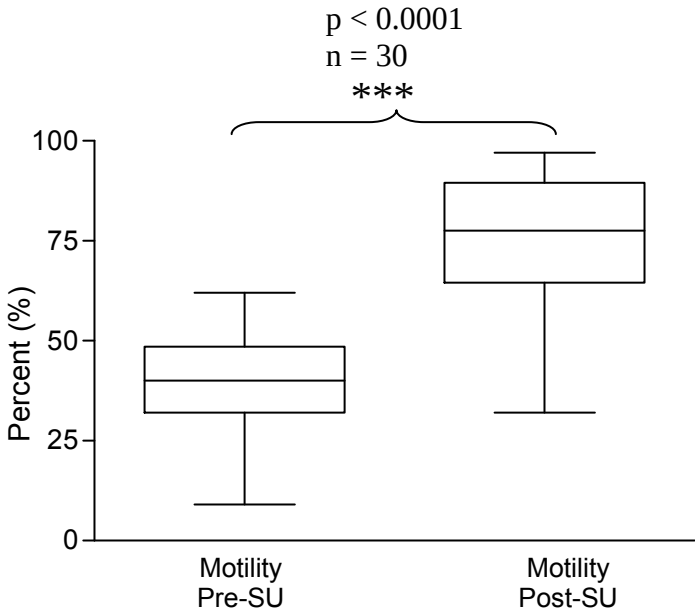


Figure 9 The degree of difference between pre- and post-swim-up motility, as shown using a box and whisker plot.

In comparison with pre-swim-up values, higher motility was observed post-swim-up in all donors. While in pre-swim-up motility results, no donors were found to possess average motility greater than 50%, the highest being the average value of 48.20% for Donor 3, post-swim-up results show that all donors displayed average motility greater than the WHO reference value of 50%[26].

CASA exhibits high sensitivity to motility and could effectively differentiate between immotile, motile but non-progressive and progressively motile spermatozoa divided into sub-populations: rapid, medium and slow moving. Motility thus includes spermatozoa that were moving and this was seen to be significantly increased in the post-swim-up population compared with the population analysed directly after ejaculation and liquefaction. It was also observed that in the post-swim-up fraction the minimum percentage motility of any given sample was higher than the maximum % motility of any in the pre-swim-up fraction.

Progressive motility:

Pre-swim-up, as shown in Table 3, Donor 7 was observed to have the highest mean progressive motility (16.6 ± 4.34%), and Donor 5 the lowest mean progressive motility (11.7 ± 4.63%). Post-swim-up, Donor 4 had the lowest mean progressive motility (10.7 ± 0.67%), while Donor 3 recorded the highest average progressive motility (35.2 ± 9.24%). The average progressive motility was significantly higher ($p = 0.0046$) for the post-swim-up fractions than for the pre-swim-up fractions (Figure 10). It was also observed that the difference between average post-swim-up and average pre-swim-up progressive motility was less than that for motility (9.54% vs. 36.53%).

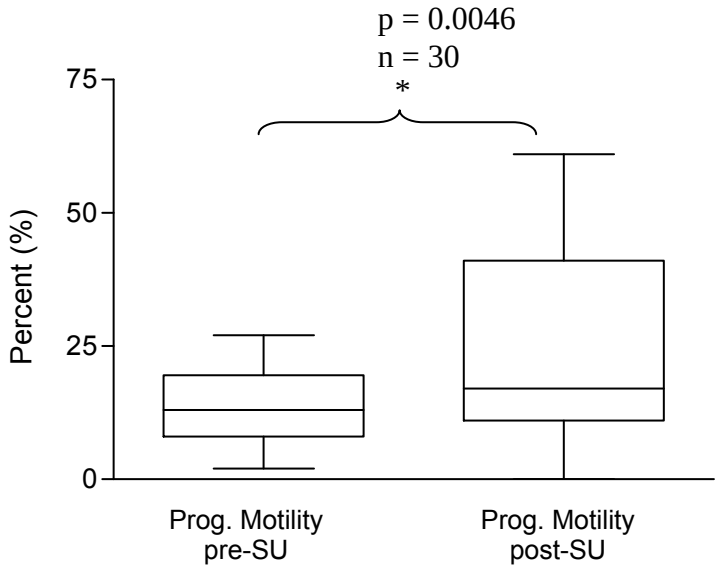


Figure 10 Box and whisker plot to indicate the degree of difference between the pre- and post-swim-up progressive motility, as measured using CASA.

For Donor 4 it was observed that post-swim-up progressive motility was lower than in than in its pre-swim-up populations (13.3 ± 5.04% vs.10.7 ± 0.67%). Six of the seven examined donors' percentage progressive motility was seen to be higher in the post-swim-up fraction as the average progressive motility increased by almost 10%.

In Figure 10 it is indicated that the post-swim-up distribution of values observed for progressive motility were greater than those observed for pre-swim-up populations, The observed significant difference between the pre- and post-swim-up values was expected since swim up selection is based on the principle that spermatozoa must swim from the pellet into the swim-up fraction, thus requiring progressive motility. The spermatozoa found in the swim-up fraction are assumed to have been sufficiently progressively mobile to reach the swim-up fraction, and many are axpected to still show progressive motility shortly after the time for swim-up selection ended. Conversely it was expected that the spermatozoa not capable of progressive motility would be seen in the pre-swim-up populations, but would not be able to enter the

post-swim-up population due to their inability to leave the pellet at the bottom of the test tube. The above expectation appears validated by the observation that there is an increase seen in the mean progressive motility for all seven donors from the pre-swim-up average of 13.5 ± 0.68% to the post-swim-up average of 23.0 ± 3.35%

Table 4 VAP, VSL and VCL average pre- and post-swim-up values for spermatozoa of 7 donors, as well as the pre- and post-swim-up averages for the groups.

`	VAP (µm/s)						VSL (µm/s)					
	n	Pre-swim-up		n	Post-swim-up		n	Pre-swim-up		n	Post-swim-up	
1	3	50.67	± 4.34	3	57.33	± 4.54	3	43.63	± 3.99	3	51.87	± 5.31
2	6	44.03	± 3.96	6	54.43	± 3.79	6	38.43	± 3.38	6	48.57	± 3.28
3	5	40.56	± 3.91	5	64.08	± 5.51	5	37.04	± 4.06	5	59.98	± 5.39
4	3	44.83	± 4.08	3	45.03	± 3.35	3	39.90	± 3.88	3	42.03	± 3.17
5	3	47.57	± 4.02	3	55.90	± 17.42	3	43.07	± 3.79	3	53.00	± 16.22
6	5	49.84	± 2.59	5	50.68	± 5.75	5	44.42	± 2.67	5	46.66	± 5.42
7	5	45.32	± 3.60	5	51.10	± 4.83	5	39.60	± 3.46	5	46.58	± 4.89
Average	**30**	**46.12**	**± 1.33**		**54.08**	**± 2.27**		**40.87**	**± 1.07**		**49.81**	**± 2.18**

Subject ID	VCL (µm/s)					
	n	Pre-swim-up		n	Post-swim-up	
1	3	66.07	± 5.37	3	69.10	± 4.45
2	6	55.33	± 4.31	6	68.57	± 4.61
3	5	47.30	± 3.76	5	74.18	± 4.99
4	3	58.30	± 2.80	3	55.30	± 2.63
5	3	58.63	± 3.92	3	63.03	± 16.09
6	5	61.88	± 2.73	5	61.08	± 5.27
7	5	59.04	± 4.03	5	59.14	± 5.45
Average	**30**	**58.08**	**± 2.20**		**64.34**	**± 2.48**

VAP

In Table 4 the highest mean VAP and highest SE pre-swim-up was recorded for Donor 1 (50.67 ± 4.34µm/s), Donor 3 was seen to have the lowest mean pre-swim-up VAP (40.6 ± 3.91 µm/s), and the highest mean post-swim-up VAP (64.1 ± 5.51 µm/s).

From Figure 11 it is seen that there was a significant difference between the average pre- and post-swim-up populations VAP measurements (p = 0.0092).

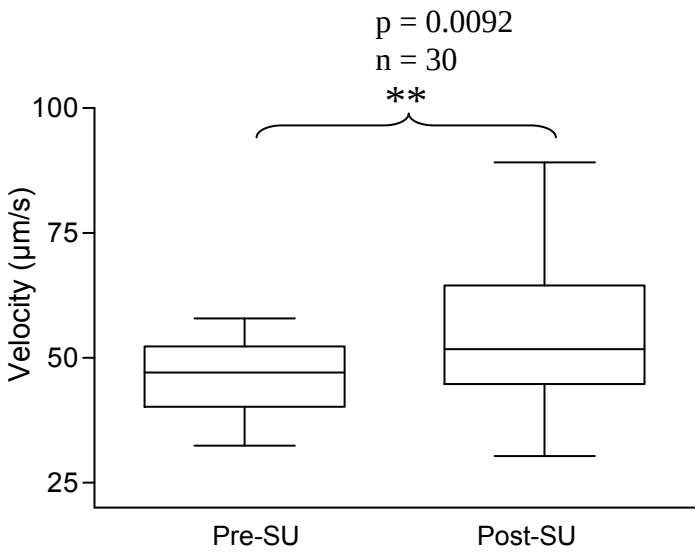


Figure 11 This Box and Whisker plot indicates the degree of difference between the measured pre- and post-swim-up VAP, as measured using CASA.

VSL

The lowest mean VSL and highest SE pre-swim-up was observed for Donor 3 (37.0 ± 4.06 µm/s), the same Donor was seen to have the highest post-swim-up mean VSL (60.0 ± 5.39 µm/s). As for the highest mean pre-swim-up VSL, this was recorded for Donor 6 (44.4 ± 2.67 µm/s). There was a significant increase (p = 0.0031) observed in the VSL of post-swim-up versus pre-swim-up populations, as can be seen in Figure 12 .

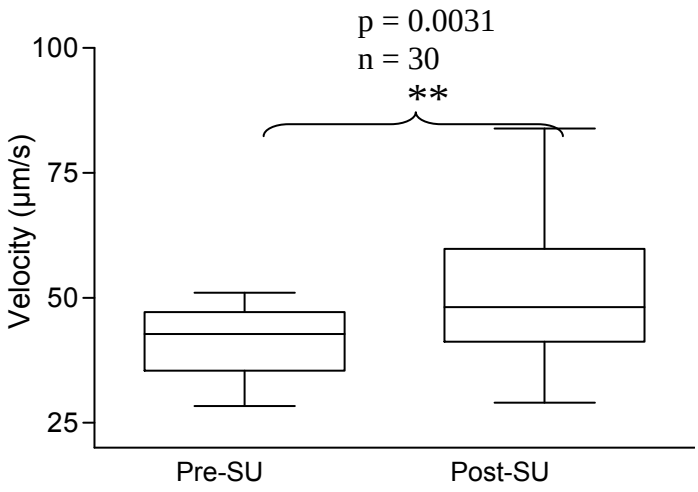


Figure 12 Plot illustrating the significantly different post-swim-up VSL measurements as analysed.

VCL

In Table 4 it is shown that Donor 1 was seen to have the highest pre-swim-up mean VCL and SE (66.1 ± 5.37 µm/s), while Donor 3 was seen to have both the lowest mean VCL pre-swim-up (47.3 ± 3.76 µm/s) as well as the highest mean VCL post-swim-up (74.2 ± 4.99 µm/s).

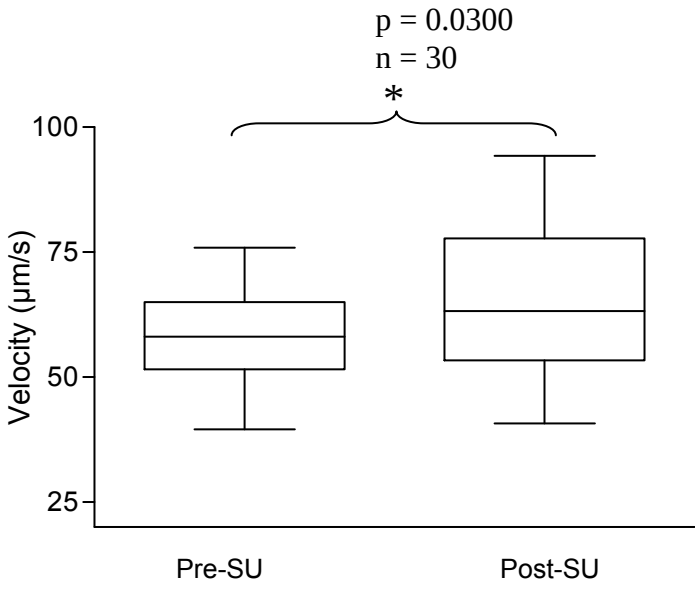


Figure 13 Plot illustrating the significantly higher post-swim-up VCL measurements as analysed using the paired *t*-test.

The mean post-swim-up VCL for Donor 4 was the lowest for the study group (55.3 ± 2.63 µm/s). The difference, seen in Figure 13 between the average pre- and post-swim-up VCL was found to be significant (p = 0.0300).

The average values of the three kinematic velocity components; VAP, VSL and VCL, as indicated in Table 4, were observed to be significantly higher for the post-swim-up group in all three cases. The average VAP and VSL values of Donor 4 showed very little increase and there was a decrease in the post-swim-up VCL value compared to the pre-swim-up average of Donor 4. Donor 6 showed the same trend with the VAP and VSL values both showing small increases in the post-swim-up average. The decrease in the post-swim-up average VCL value was, however, only marginal (0.8 µm/s) in case of Donor 6. Other than the abovementioned decrease in average VCL observed for Donor 4 and 6, post-swim-up all average values were higher for VAP, VSL and VCL as recorded in Table 4.

VAP, VSL and VCL was significantly higher post-swim-up, the differences were represented by p values of 0.0092, 0.0031 and 0.0300, respectively. This observed increase in the kinematic parameters was associated with the movement of the spermatozoa from the pellet up to the fraction which was analysed as the swim-up fraction. The VAP, VSL and VCL properties enable the spermatozoa to complete swim-up at a rate directly proportional to their level of kinematics.

ALH

The highest pre-swim-up value recorded in Table 5 was for Donor 1 (7.47 ± 0.41 µm). Donor 3 had the lowest mean pre-swim-up ALH (3.46 ± 0.19 µm) and Donor 5 was recorded as having the lowest post-swim-up mean ALH, as well as

the highest SE (2.97 ± 1.48 µm). The mean post-swim-up ALH for Donor 2 had the largest average recorded of all seven the donor's post-swim-up ALH values (5.88 ± 1.07 µm). The decrease seen in mean ALH post swim up for this group as a whole was not significant (p = 0.0623).

Table 5 Results of the measured kinematic parameters: amplitude of lateral head movement and beat cross frequency of spermatozoa pre- and post-swim-up.

Subject ID	ALH (µm)				BCF (Hz)			
	n	Pre-swim-up	n	Post-swim-up	n	Pre-swim-up	n	Post-swim-up
1	3	7.47 ± 0.41	3	5.47 ± 0.49	3	6.57 ± 0.28	3	7.73 ± 0.86
2	6	5.03 ± 0.26	6	5.88 ± 1.07	6	6.03 ± 0.20	6	8.47 ± 0.11
3	5	3.46 ± 0.19	5	5.04 ± 0.60	5	5.70 ± 0.14	5	8.20 ± 0.31
4	3	6.20 ± 0.59	3	4.57 ± 0.64	3	7.07 ± 0.23	3	8.53 ± 0.55
5	3	5.90 ± 0.10	3	2.97 ± 1.48	3	6.27 ± 0.24	3	7.37 ± 0.52
6	5	5.68 ± 0.24	5	4.34 ± 0.68	5	6.88 ± 0.18	5	8.58 ± 0.49
7	5	6.26 ± 0.32	5	3.94 ± 0.45	5	6.02 ± 0.28	5	7.88 ± 0.35
Average	**30**	**5.71 ± 0.47**	**30**	**4.60 ± 0.37**	**30**	**6.36 ± 0.19**	**30**	**8.11 ± 0.17**

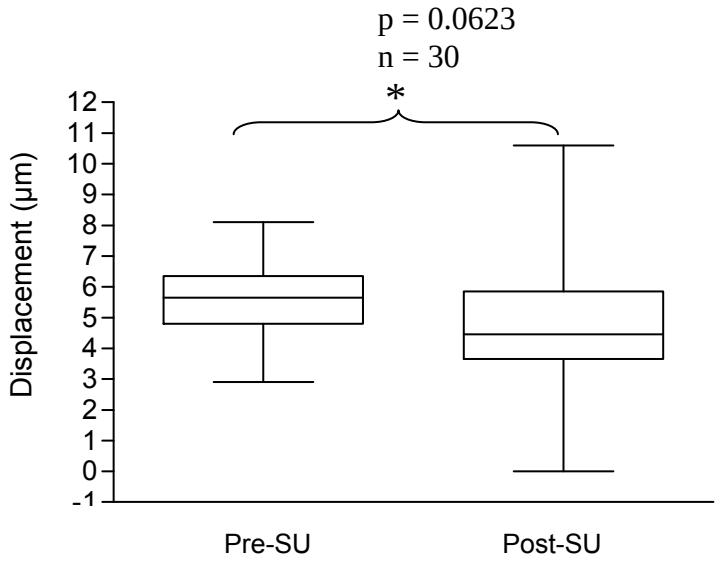


Figure 14 An indication of the degree of decrease for the measured parameter ALH pre- and post-swim-up as indicated by the paired *t*-test.

Donor 3 had the lowest mean pre-swim-up ALH, and was observed to be the only donor to record an average ALH lower than 4 µm. post-swim-up this average increased (5.04 ± 0.60 µm), this was contrary to the trend observed as five of the remaining six donors (apart from Donor 5) showed a decrease in ALH post-swim-up.

Swim-up was seen to have a strong selecting effect with regard to some sampled movement characteristics. ALH however, was an exception in that post-swim-up there was no significant difference in ALH. Where an increase had been observed in average motility, progressive motility, VAP, VSL, VCL, and as will be discussed shortly, BCF, there was no increase in the ALH, on the contrary the average post-swim-up ALH for the donors was lower than the observed means pre-swim-up. Tthis could possibly be linked to the more rapid and frequent beating of the tail, such that the head is pushed to and fro more rapidly and there is thus less time to travel far to the one side, resulting in an increased ALH, such as that observed for the slower, less motile pre-swim-up populations displaying lower BCF (Table 5).

BCF

In Table 4 the highest frequency pre-swim-up was recorded by Donor 4 (7.07 ± 0.23 Hz), the second highest being observed for Donor 6 (6.88 ± 0.18 Hz). Donor 3 was seen to have the lowest mean BCF (5.70 ± 0.14 Hz). Post-swim-up Donor 6 was seen to have the highest mean frequency (8.58 ± 0.49 Hz), while the post-swim-up mean BCF for Donor 4 was the second highest (8.53 ± 0.55 Hz). Donor 5 averaged the lowest post-swim-up BCF (7.37 ± 0.52 Hz). It was observed that there was a significant increase in BCF post-swim-up ($p < 0.001$; Figure 15) for the group as a whole.

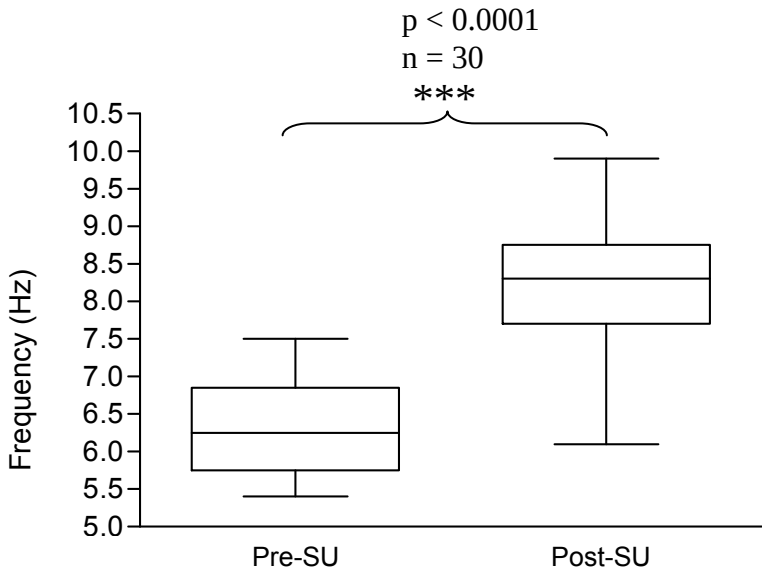


Figure 15 The Box and Whisker Plot indicates the significant increase in BCF post-swim-up in comparison with the paired pre-swim-up measurements.

This significant increase in BCF is in keeping with the rationale of swim-up-selection, which is to separate spermatozoa capable of self propulsion from those that are immotile and would therefore be unable to naturally reach the oocyte.

3.3.3 Morphometry

The morphometrical results of the smears of spermatozoa from the respective samples are shown in Table 6. The stained slides were analysed with morphometrical software as described in the materials and methods chapter, and the results recorded. The average morphometrical values determined for each donor was shown with the population averages indicated in the bottom row of Table 6. The ASMA system uses the combined morphometrical data obtained to classify spermatozoa as either normal or abnormal. Measured parameters such as spermatozoa head area were then presented with a value for the normal

spermatozoa and a separate value for the abnormal spermatozoa. In order to then calculate the means for the pre- and post-swim-up populations, this was taken into account. These calculated population means were illustrated in Table 6.

If, for example, 20% of an analysed population was determined normal, the average values for acrosome size of the normal subpopulation then contributes 20% and the average measurements of head size for the abnormal subpopulation 80% to the population average displayed in the bottom row of the tables illustrating morphometrical data.

It should be noted that due to experimental complications such as under- or over-staining of slides, decondensation of spermatozoa membranes and swim-up concentrations below the analysis threshold, six of the 30 pre-swim-up samples did not stain correctly. The ASMA software was subsequently unable to analyse these slides due to the low number of spermatozoa left in the fields analysed by the system. The number of data sets for the pre-swim-up samples were thus reduced from 30 to 24.

Spermatozoa head area

The highest mean pre-swim-up head area and SE was seen for Donor 1 (13.13 ± 1.71 μm^2), while the lowest was recorded for Donor 7 (9.96 ± 0.997 μm^2). Post-swim-up, Donor 6 recorded the highest mean area (13.17 ± 0.618 μm^2), Donor 3 was observed to have the lowest mean area (11.37 ± 0.449 μm^2).

Table 6 illustrates that the average spermatozoa head area values for Donors 1 and 3 showed an average decrease, with the average decrease in Donor 3 being the largest from pre-swim-up to post-swim-up values.

Table 6 Results of morphometric analysis of spermatozoa pre- and post-swim-up, examining the spermatozoan head area and acrosomal properties.

Subject ID	Spermatozoa head area (μm^2)							Acrosome size (μm^2)						
	n	Pre-swim-up			n	Post-swim-up			n	Pre-swim-up			n	Post-swim-up		
1	2	13.13	±	1.705	3	12.67	±	0.504	2	3.53	±	0.252	3	4.08	±	0.163
2	5	11.37	±	0.284	6	11.69	±	0.505	5	3.93	±	0.104	6	3.67	±	0.171
3	4	12.58	±	0.322	5	11.37	±	0.449	4	3.87	±	0.240	5	3.68	±	0.095
4	2	11.35	±	0.105	3	12.65	±	1.037	2	4.21	±	0.095	3	4.44	±	0.311
5	3	10.79	±	0.777	3	12.41	±	0.521	3	4.00	±	0.203	3	3.92	±	0.285
6	4	12.63	±	0.433	5	13.17	±	0.618	4	4.74	±	0.257	5	3.96	±	0.473
7	4	9.96	±	0.997	5	12.03	±	1.095	4	3.90	±	0.390	5	4.49	±	0.359
Average	**24**	**11.68**	**±**	**0.430**	**30**	**12.28**	**±**	**0.240**	**24**	**4.03**	**±**	**0.142**	**30**	**4.03**	**±**	**0.125**

Subject ID	Acrosome %							Acrosome Index (%)						
	n	Pre-swim-up			n	Post-swim-up			n	Pre-swim-up			n	Post-swim-up		
1	2	27.76	±	5.317	3	32.13	±	0.127	2	25.54	±	3.314	3	30.94	±	4.560
2	5	34.62	±	1.246	6	31.51	±	1.758	5	39.75	±	8.542	6	27.03	±	4.375
3	4	30.99	±	2.143	5	32.62	±	1.995	4	28.79	±	6.308	5	25.42	±	7.145
4	2	37.15	±	1.105	3	35.23	±	0.445	2	42.81	±	0.945	3	34.92	±	3.497
5	3	37.04	±	0.757	3	31.47	±	1.001	3	41.11	±	4.258	3	24.92	±	4.236
6	4	37.52	±	1.614	5	30.87	±	4.806	4	44.11	±	5.945	5	33.51	±	13.132
7	4	39.21	±	0.750	5	37.61	±	1.027	4	53.91	±	1.586	5	42.80	±	4.307
Average	**24**	**35.19**	**±**	**0.929**	**30**	**33.06**	**±**	**0.928**	**24**	**39.43**	**±**	**3.625**	**30**	**31.36**	**±**	**2.409**

All other donors showed an increase in average head area. It was noted that the highest means pre- and post-swim-up were not much different (13.13 μm^2 for Donor 1 and 13.17 μm^2 for Donor6), however the two lowest values recorded pre-swim-up (9.96 μm^2 for Donor 7 and 10.79 μm^2 for Donor 5) were significantly lower than the pre-swim-up maximum, while post-swim-up the two lowest values (11.37 μm^2 for Donor 3 and 11.69 μm^2 for Donor 2) were nearer the maximum.

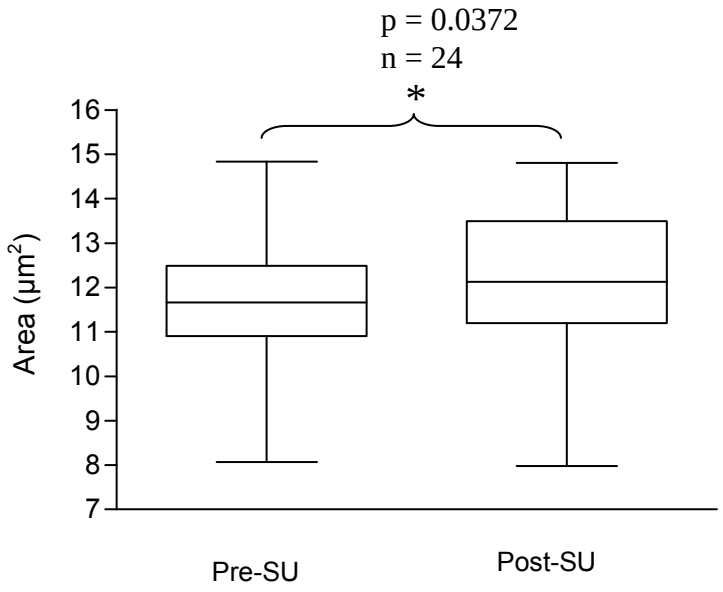


Figure 16 Box and whisker plot to indicate the degree of difference between Spermatozoa head area, pre- and post-swim-up.

A significantly different average, as seen in Figure 16 , for the post-swim-up group was obtained and the mean increase for the seven donors was from 11.68 ± 0.43 μm^2 pre-swim-up to 12.28 ± 0.24 μm^2 post-swim-up. For the pre-swim-up populations the measurements were very evenly distributed, while post-swim-up, as can be seen in Figure 16 the distribution was not as even.

Acrosome size

Pre-swim-up, Donor 6 was seen to record the highest mean acrosome size (4.74 ± 0.257 μm^2), the lowest mean acrosome size was observed for Donor 1 (3.53 ± 0.252 μm^2). Post-swim-up Donor 7 was observed to have the highest mean acrosome size (4.49 ± 0.359 μm^2), and Donor 2 recorded the lowest mean acrosome size (3.67 ± 0.171 μm^2).

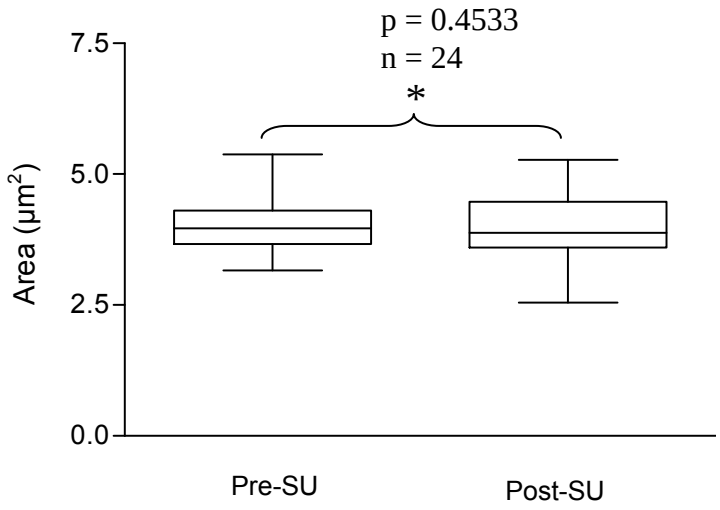


Figure 17 Box and whisker plot to indicate the lack of significant difference between acrosome size pre- and post-swim-up.

The mean for both the pre- and post-swim-up donors average acrosome size was 4.03 μm^2 (pre-swim-up SE was ± 0.142 μm^2, while post-swim-up SE was ± 125 μm^2). The acrosome size was found not to differ significantly between the pre-swim-up and post-swim-up populations ($p = 0.4533$). This agrees with the findings of published research[156].

The area of the acrosome was calculated by finding the product of the spermatozoa head area and the acrosome %, both of which were directly measured by the ASMA hardware and calculated using the software algorithms. The acrosome size was thus

an indirect measurement. It can be seen that the highest mean pre-swim-up value (in this case for Donor 6) was greater than the highest mean post-swim-up value recorded (Donor 7), while the smallest mean value was seen in the pre-swim-up group (Donor 1). It was observed that although the post-swim-up head area was significantly larger, the acrosome size was, on average, unchanged post-swim-up. This can only be due to the acrosome % displaying the opposite trend to that observed for head area.

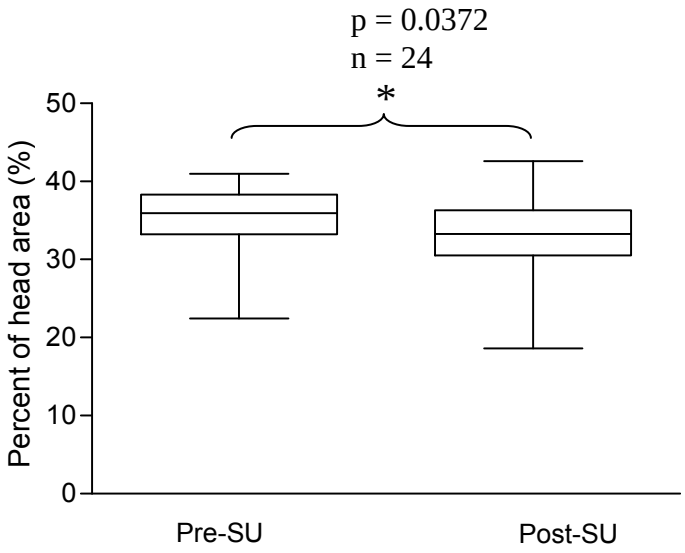


Figure 18 Box and whisker plot to indicate the degree of difference between pre- and post-swim-up acrosome percentage.

Acrosome %

The average acrosome % for the pre-swim-up group was 35.2 ± 0.929%, with the average for the post-swim-up group having been 33.1 ± 0.928%. Donor 1 was seen to have the lowest mean acrosome % (27.76%), with Donor 7 recording the highest mean pre-swim-up (39.21 ± 0.750%) and post-swim-up (37.61 ± 1.027%) acrosome %. The lowest post-swim-up acrosome % was observed for Donor 6 (30.87%) who also recorded the highest post-swim-up SE (± 4.806%). Post-swim-up measurements

for acrosome % indicated a significantly decrease in this parameter compared to the measured pre-swim-up values (p = 0.037; Figure 18).

In contrast to the trend for a significantly larger head area post-swim-up, the relative contribution of the acrosome to the head area (Acrosome %) decreased post-swim-up and was observed to differ significantly, as illustrated in Figure 19 , from the acrosome % of the pre-swim-up population (p = 0.037). The acrosome % was seen to be reduced post-swim-up by an average of 2.12% from 35.19% to 33.06%, this translated into a decrease of approximately six percent. This would suggest that on average most sampled spermatozoa did not meet the criteria for being classified as “normal”, set by the 1999 WHO laboratory manual requiring the acrosomal size to cover 40-70% of the distal part of the sperm head. The 1992 WHO criteria would, however, have been met since that publication required a minimum of 33% and not 40-70% [26].

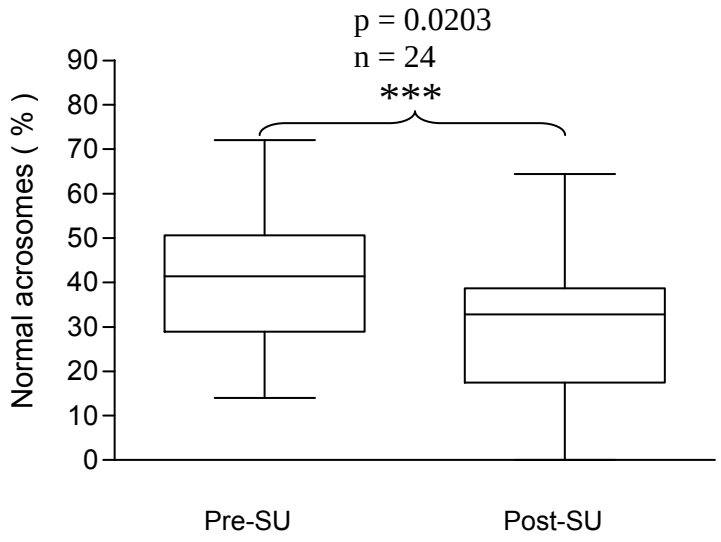


Figure 19 Box and whisker plot to indicate the degree of difference between acrosome index pre- and post-swim-up.

AI

Donor 1 recorded the lowest mean pre-swim-up AI (25.53 ± 3.314%) while Donor 7 recorded the highest mean pre-swim-up (53.91 ± 1.586%) and post-swim-up AI (42.80 ± 4.307%). The lowest mean AI recorded post-swim-up was for Donor 5 (24.92 ± 4.307%. The pre-swim-up average for the group was 39.43 ± 3.625%, decreasing significantly to 31.36 ± 2.409% post-swim-up ($p = 0.0203$, Figure 19)

Donor 1 was the only one to show an increase in mean AI from pre- to post-swim-up. All six the other donors were seen to record a lower value for AI post-swim-up, Donor 5 was being observed to have the largest mean decrease, from 41.11 ± 4.258 to 24.92 ± 4.236.

Manual morphology analysis

The results of this analysis was recorded in Table 1 , it was observed that the morphology of the group was limited to a maximum of 12% normal (Donors 4 and 6) and a minimum of 8% normal spermatozoa (Donor 1). The analysis was only done once for each donor, since it was used to obtain an initial indication of the morphology, which may then be compared to morphology as assessed automatically, had this assessment become part of the protocol.

3.4 ACROSOME SIZE CORRELATIONS

3.4.1 Variation in the sample size

Table 7 represents the statistical results of the correlation between pre-swim-up AS and measured variables and parameters while in Table 8 correlation analyses of the same variables with post-swim-up average AS is presented. The sample sizes are smaller in the pre-swim-up table (Table 7) since only 24 pre-swim-up morphometric samples stained sufficiently to be analysed accurately by the ASMA system.

3.4.2 Pre-swim-up AS correlations

The correlation of pre-swim-up AS was not significant with the following pre-and post-swim-up parameters: concentration, motility, progressive motility, morphology, VAP, VSL, VCL and AI (Table 7). Post-swim-up motility and pre-swim-up AS showed the highest degree of correlation (Pearson r = 0.394), however, with an r^2 value of 0.155 pre-swim-up AS was found to not predict post-swim-up motility.

It was noted that there was no significant positive correlation (Table 7) between pre-swim-up AS and pre-swim-up progressive motility, VAP, VSL or VCL, while the correlation between pre-swim-up AS and post-swim-up kinematic parameters, though not significant, becomes negative in the cases of post-swim-up progressive motility, VAP, VSL and VCL. The reason for the negative correlations with the post-swim-up kinematic variables progressive motility, VAP, VSL and VCL may be due to the greater resistance and inertia present in motile spermatozoa with larger acrosomes.

Table 7 The degree of correlation observed between pre-swim-up AS and kinematic variables.

Average AS pre-swim-up vs.	Concentration (M/ml)		Motility (%)		Progressive motility (%)	
	Pre-swim-up	Post-swim-up	Pre-swim-up	Post-swim-up	Pre-swim-up	Post-swim-up
(n)	24	24	24	24	24	24
p value (two-tailed)	0.379	0.611	0.973	0.0571	0.469	0.241
Pearson r	-0.188	-0.109	0.00718	0.394	0.155	-0.249
r^2	**0.0354**	**0.0120**	**0.0000516**	**0.155**	**0.0241**	**0.0619**

Average AS pre-swim-up vs.	VAP (µm/s)		VSL (µm/s)		VCL (µm/s)	
	Pre-swim-up	Post-swim-up	Pre-swim-up	Post-swim-up	Pre-swim-up	Post-swim-up
(n)	24	24	24	24	24	24
p value (two-tailed)	0.284	0.140	0.275	0.140	0.410	0.0659
Pearson r	0.228	-0.310	0.232	-0.310	0.176	-0.381
r^2	**0.0520**	**0.0962**	**0.0539**	**0.0961**	**0.0310**	**0.146**

Table 8 Degree of correlation observed between post-swim-up AS and kinematic variables.

Average AS post-swim-up vs.	Concentration (M/ml)		Motility (%)		Progressive motility (%)	
	Pre-swim-up	Post-swim-up	Pre-swim-up	Post-swim-up	Pre-swim-up	Post-swim-up
(n)	30	30	30	30	30	30
p value (two-tailed)	0.195	0.592	0.521	0.0257	0.519	0.0991
Pearson r	-0.243	0.102	-0.122	0.407	0.123	-0.307
r^2	**0.0593**	**0.0104**	**0.0149**	**0.165**	**0.0150**	**0.0942**

Average AS post-swim-up vs.	VAP (µm/s)		VSL (µm/s)		VCL (µm/s)	
	Pre-swim-up	Post-swim-up	Pre-swim-up	Post-swim-up	Pre-swim-up	Post-swim-up
(n)	30	30	30	30	30	30
p value (two-tailed)	0.443	0.0330	0.475	0.0449	0.362	0.00800
Pearson r	0.151	-0.390	0.141	-0.369	0.179	-0.475
r^2	**0.0228**	**0.152**	**0.0198**	**0.136**	**0.0320**	**0.226**

The relationship may be indirect, i.e. the large average pre-swim-up AS in a population leading to large post-swim-up AS which, due to fluid resistance kinetics, in turn results in a decrease in the measured kinematic variables. This reasoning was supported by the significant negative correlations seen between post-swim-up AS and post-swim-up VAP, VSL and VCL (Table 8).

3.4.3 Post-swim-up AS correlations

Significant negative correlations were observed between average post-swim-up AS and three post-swim-up kinematic parameters: VAP post-swim-up, VSL post-swim-up and VCL post-swim-up (Table 8). These negative correlations translate into a linear decrease in the magnitude of these measured kinematic parameters for an increase in post-swim-up AS. The r^2 values indicate that for these four parameters between 13.6% and 22.6% of the variance in the kinematic variables was due to their post-swim-up AS. Though this percentage was reasonably small, it was postulated that during the progression of spermatozoa through the swim-up medium, resistance kinetics associated with larger acrosomes translated into the observed significant decrease in the magnitude of VAP, VSL and VCL seen for populations with greater average AS.

As a whole, there was no significant correlation between pre-swim-up AS and any of the selected parameters as shown in (Table 8). In contrast, post-swim-up AS appeared to partially determines the post-swim-up kinematics in the form of VAP, VCL and VSL.

3.5 SIGNIFICANT KINEMATIC CORRELATIONS WITH ACROSOMAL SIZE

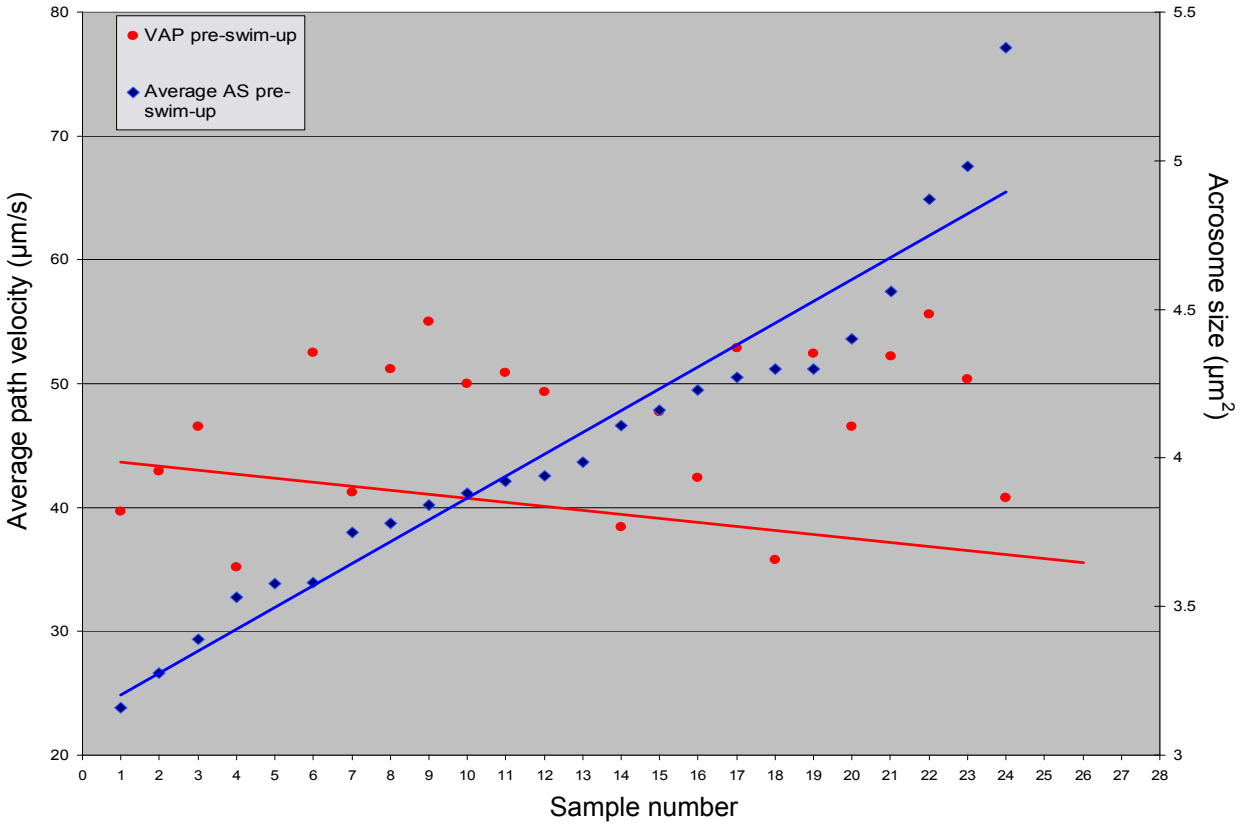


Figure 20 Change in VAP pre-swim-up against increasing average AS pre-swim-up.

Figure 20 illustrates that there was no significant inverse relationship seen in the data trend of VAP pre-swim-up with increasing pre-swim-up AS. This observation was deduced from the low correlation (r^2 = 0.052) between these two parameters illustrated in Table 7 . Since the analysis was done on ejaculated spermatozoa that did not necessarily need to be progressively motile to be included in the analysed population the AS had no opportunity to influence the kinetics and motion and subsequently the location of the analysed cells. As a result the low correlation was expected.

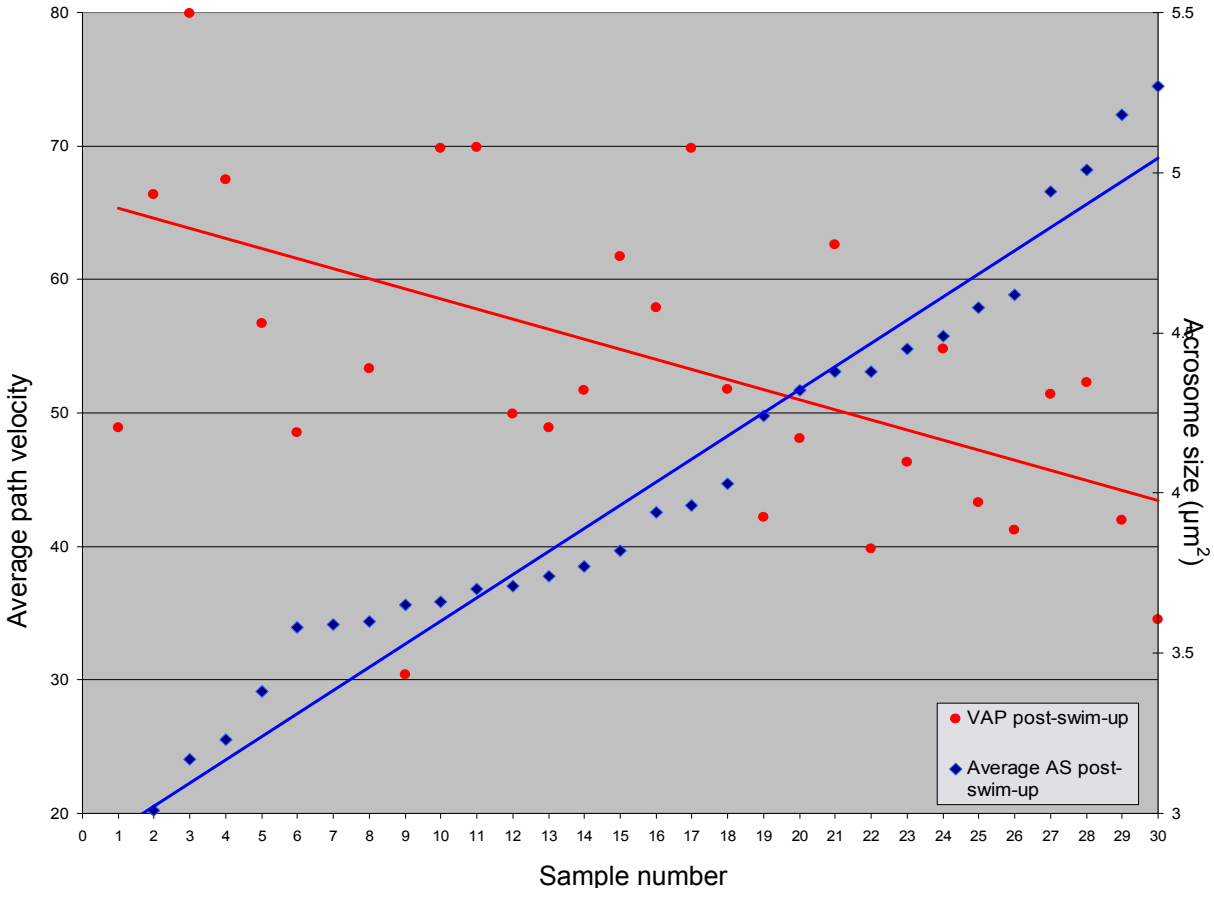


Figure 21 Correlation of average AS post-swim-up with VAP post-swim-up.

Figure 21 illustrates the absence of any significant relationship observed between pre-swim-up AS and pre-swim-up VAP. Once swim-up selection was applied, the post-swim-up AS clearly showed an inverse relationship as evidenced by the significant negative correlation ($p = 0.0330$, $r = -0.390$ and $r^2 = 0.152$) with post-swim-up VAP (Table 8). As post-swim-up AS increased, the observed VAP of the post-swim-up population was seen to decrease.

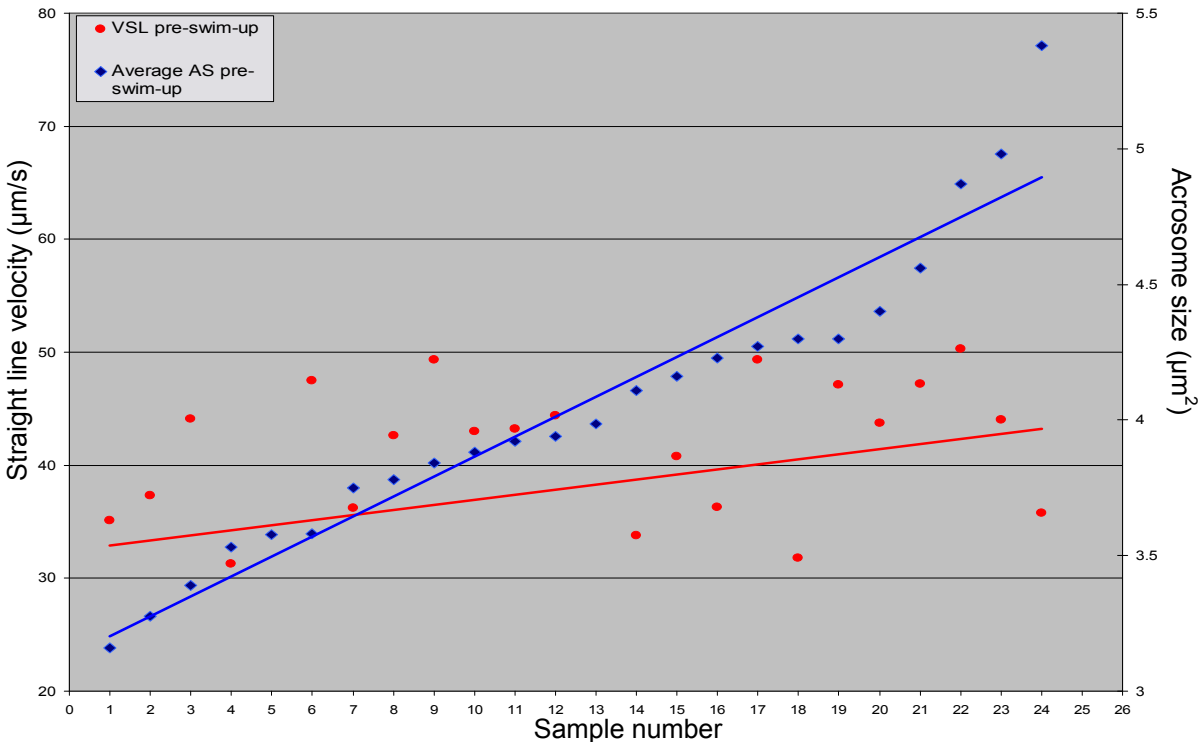


Figure 22 Change in with VSL pre-swim-up plotted against increasing average AS pre-swim-up.

Figure 22 and Figure 23 showed the same trend as seen for VAP since the observed decrease in the post-swim-up kinematic parameter, in this case VSL post-swim-up, was significantly correlated ($p=0.0449$, $r=-0.369$ and $r^2=0.136$) with increased post-swim-up AS. Pre-swim-up AS had not showed any significant correlation with pre-swim-up VSL ($r^2=0.054$) of with post-swim-up VSL ($r^2=0.096$), and post-swim-up VSL had not showed a significant relationship with post-swim-up AS ($r^2=0.020$).

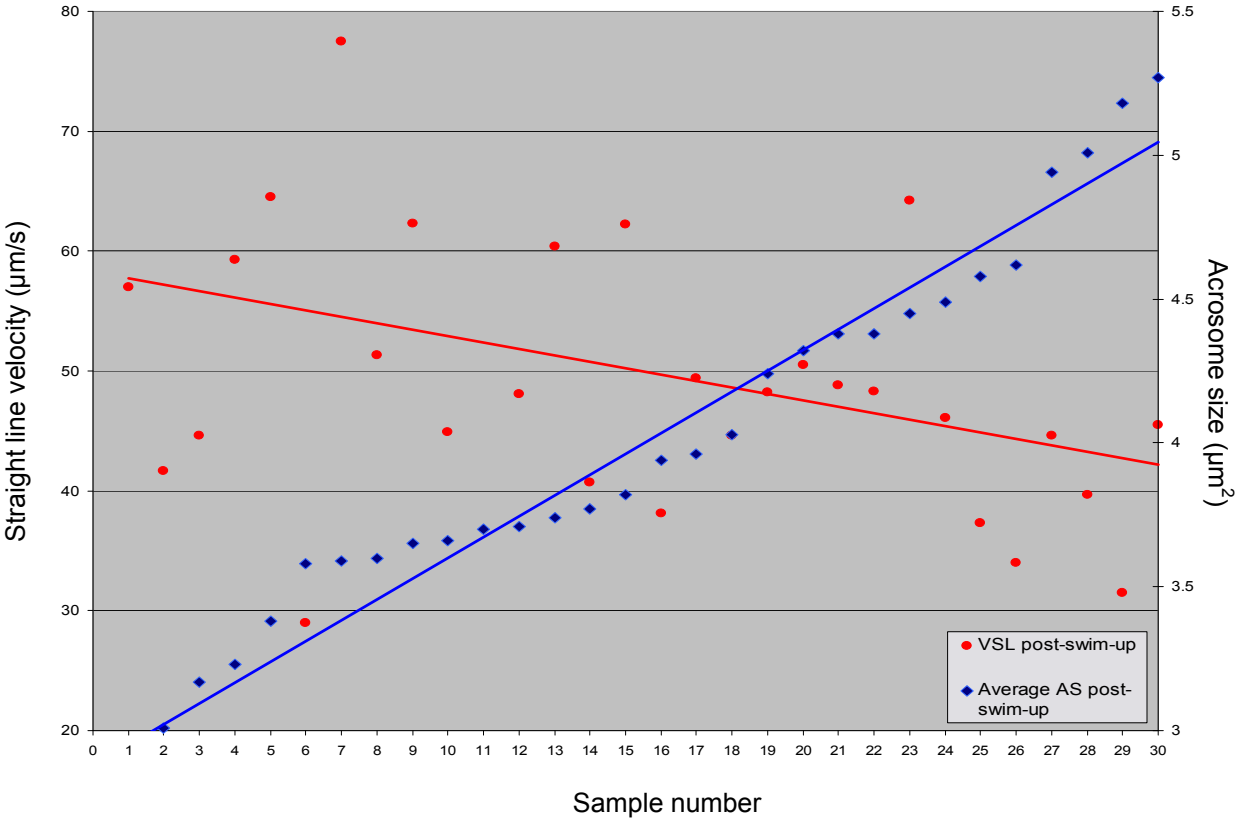


Figure 23 Correlation of average AS post-swim-up with VSL post-swim-up.

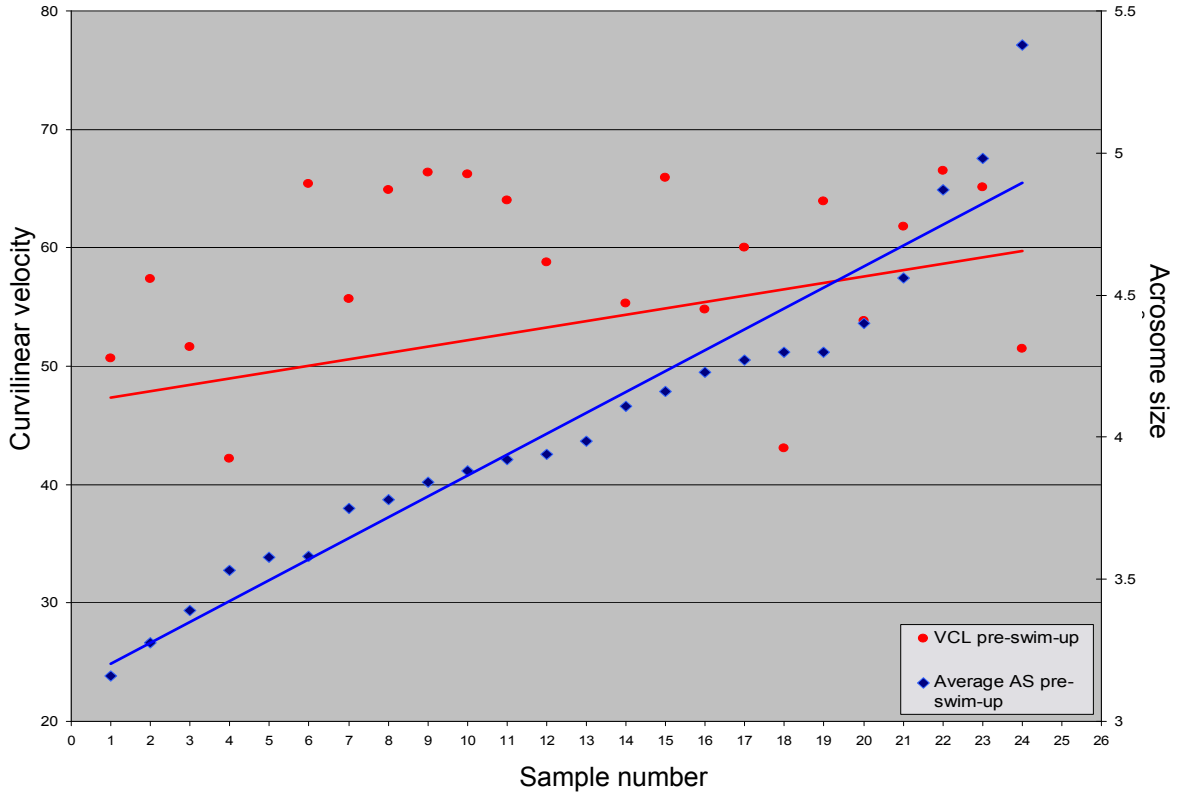


Figure 24 Change in VCL pre-swim-up plotted against increasing average AS pre-swim-up.

Figure 22 and Figure 24 show that, as in the case of the correlation with pre-swim-up VSL (p=0.475; r^2=0.054), there was no significant correlated change in pre-swim-up VCL (p=0.362; r^2=0.031)) that could be associated with a change in the pre-swim-up AS. The almost horizontal red line with a positive gradient in both cases represents the variable's data trend which shows no significant positive change.

The lack of change seen in the abovementioned two kinematic parameters may be due to the following reason: influence on kinematic parameters due to AS could only take place in an environment where a selection pressure was exerted on the spermatozoa through some action resulting from the properties of the acrosome. As the fresh semen does not represent such an environment, no correlation was expected to be found with the pre-swim-up kinematic parameters.

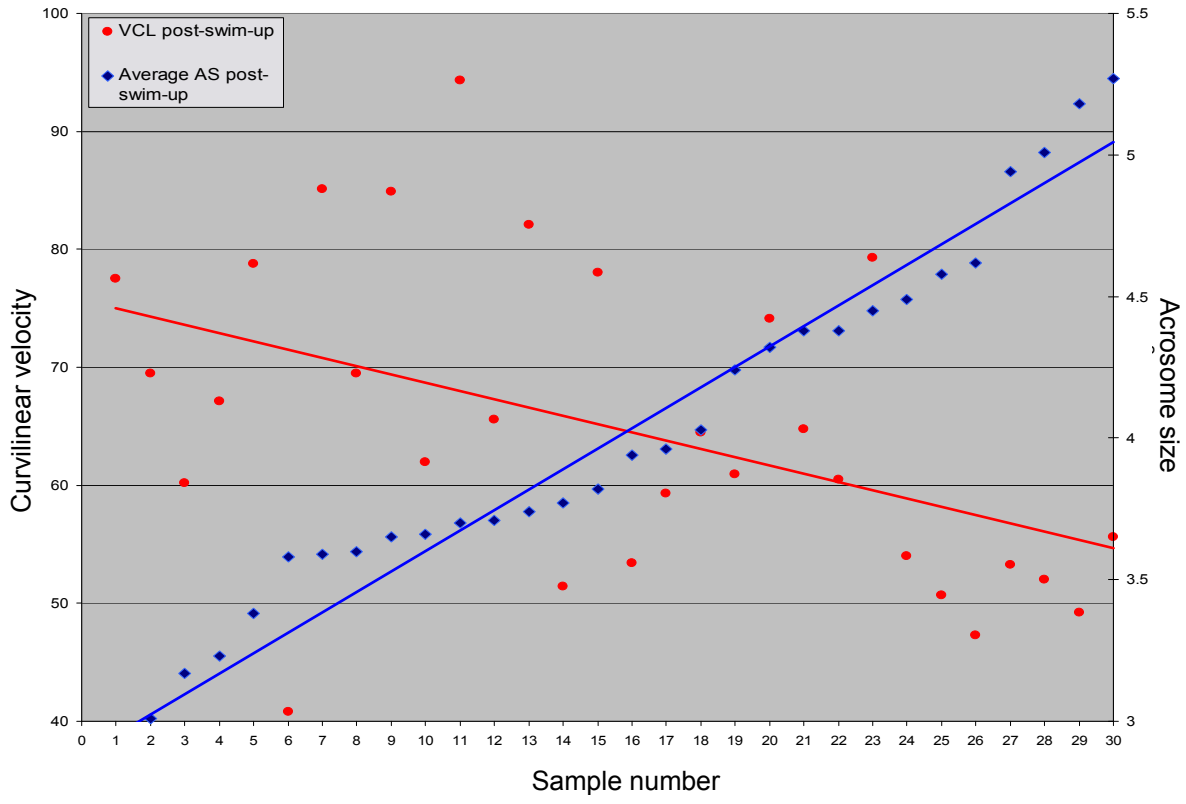


Figure 25 Correlation of average AS post-swim-up with VCL post-swim-up.

Once more in Figure 25 it is shown that there was a significant correlated decrease ($p = 0.008$, $r = -0.475$, $r^2 = 0.226$) in post-swim-up curvilinear velocity in those samples with larger acrosomes, when compared to samples with smaller acrosomes.

It was observed that irrespective of AS, higher VAP, VSL and VCL was observed post-swim-up. Pre-swim-up, these three kinematic parameters were not associated with specific acrosomal sizes, after swim-up, however, a clear inverse relationship between the AS and the abovementioned kinematic parameters was observed. Larger post-swim-up average AS was correlated to lower VAP, VSL and VCL post-swim-up.

CHAPTER 4
DISCUSSION

With the development and routine implementation of assisted reproductive techniques such as intracytoplasmic sperm injection (ICSI)[157] and pre-implantation genetic diagnosis[158] a dramatic increase in the knowledge of human gamete biology and reproductive medicine has been witnessed. This has followed extensive basic scientific research and the expanding use of assisted reproductive technologies. The use of ICSI to successfully treat male factor fetrility has provided a unique means of allowing couples diagnosed with severe male infertility to achieve their reproductive goals even in the face of previously insurmountable obstacles such as immotile sperm or samples showing high degrees of DNA damage[159].

The implementation of ICSI, however, does not come without challenges. Despite the great therapeutic advantages of this technique, ICSI provides clinicians with treatment for infertility conditions often in the absence of an etiological or pathophysiologic diagnosis. In the face of these new techniques, several questions obviously arise including (i) what are the diagnostic steps that should be used to direct infertile men to a specific therapeutical modality? (ii) when is ICSI indicated in preferance to less expensive but possibly less effective treatments? and (iii) which properties of spermatozoa are most important for successful ICSI treatment, guiding selection of ideal sperm to be selected for performing the procedure?

Due to these questions the importance of semen analyses and sperm preparation became debatable. It was proposed that all infertile couples could be helped by

applying the ICSI technique. Whatever the origin and maturity of the spermatozoa and whatever the concentration, morphology or motility qualities, fertilization would be made possible with ICSI. The concept developed that only a few sperm cells are needed to obtain not only fertilization, but also normal embryonic development and a healthy pregnancy[160]. This approach has inherent challenges however, for example, this approach does not necessitate investigation into possible abnormalities of sperm viability, transport and retention in either the male or female partner, or acquired causes that may have been treatable at a fraction of the cost may be missed. In addition associated conditions and their causes that are indicated by abnormal findings in semen analysis would not be diagnosed.

Fortunately, this strategy has been abandoned, among other reasons are that the cost in skills and finances is very high in relation to other assisted reproductive options, and the evaluation and preparation of human sperm samples for assisted reproductive programmes has regained its status in light of its important and informative value. Analyzing the quality of the sperm sample and subsequent motile cells separation ability, will define the type of assisted reproductive technique suggested for a couple.

Currently, the basic semen analysis should include the assessment of physical semen characteristics (volume, pH agglutination and viscosity), evaluation of sperm concentration, progressive motility, normal morphology[109] and viability, presence of leukospermia and immature sperm cells, detection of antisperm antibodies and a bacteriologic investigation[161]. If abnormalities are found during the basic investigation, the workup should progress to the next level: the examination of

specific sperm functions by means of what is described by the WHO as functional tests[108].

Four categories of tests have been proposed as components of this second level: (i) computer-assisted evaluation of sperm motion characteristics (CASA), (ii) inducibility of the acrosome reaction, and bio-assays that sequentially assess gamete interaction including (iii) sperm-zona pellucida binding tests and (iv) sperm-hamster egg penetration assay[162, 162, 163].

Different laboratories have highlighted the diagnostic power of these functional tests[108]. However, as discussed in the 1996 Consensus Workshop in Advanced Andrology[164] it was concluded that better standardization of CASA methods and acrosome reaction[164] techniques should be implemented prior to the introduction of these diagnostic tools in routine clinical practise, and indeed in the decade thereafter this has been actively pursued, and to some degree, achieved as implied by the use of CASA systems today[164]..Importantly, among the bio-assays of sperm-egg interaction, it was concluded that because of the powerful evidence for prediction[165] of both fertilization[166] and its failure in the IVF setting[167], sperm-zona binding tests should be favoured among the functional assays[164].

We understand that sperm dysfunction is one of the most common single causes of infertility yet, remarkably, our knowledge of the cellular and biochemical basis for this condition is very limited[168]. Indeed, our understanding of the physiology of the normal human spermatozoon, let alone the dysfunctional spermatozoon, is elementary[169].

The Consensus Workshop in Advanced Andrology[164] recommended the standardization and development of the acrosome reaction and sperm kinetics prior to its introduction as routine tools. The present study aimed to investigate the outcome of fundamental kinematics in a group of sperm donors following double wash swim-up procedures which increase the number of morphologically normal spermatozoa[157]. It is known that in the successful fertilization of oocytes by spermatozoa, a set of functionally normal parameters with regards to both the oocytes and the maturity of the spermatozoa is both mandatory and of paramount importance [170].

Since the introduction of ASMA systems it has been possible to analyse acrosome size with increasing speed and precision as well as a multitude of other morphometrical and motility parameters. Each analysed parameter was individually correlated to all the analysed parameters.

In Table 3 it is indicated that the post-swim-up distribution of values observed for progressive motility were greater than those observed for pre-swim-up populations. The observed significant difference between the pre- and post-swim-up values was expected since swim-up selection is based on the principle that spermatozoa must swim from the pellet into the swim-up fraction, thus requiring progressive motility. The spermatozoa found in the swim-up fraction are assumed to have been sufficiently progressively motile to reach the swim-up fraction, and many are expected to still show progressive motility shortly after the time for swim-up selection has ended.

Conversely it was expected that non-progressively motile spermatozoa would be seen in the pre-swim-up populations, but would not enter the post-swim-up population due to their inability to leave the pellet at the bottom of the test tube. The above possibility is supported by the observation that in addition there is an increase seen in the mean progressive motility for the seven donors from the pre-swim-up average of 13.5 ± 0.68% to the post-swim-up average of 23.0 ± 3.35%.

VAP, VSL and VCL was significantly higher post-swim-up, the differences were represented by p values of 0.0092, 0.0031 and 0.0300, respectively. This observed increase in the kinematic parameters was associated with the movement of the spermatozoa from the pellet up to the fraction which was analysed as the swim-up fraction. The VAP, VSL and VCL properties enable the spermatozoa to complete swim-up at a rate directly proportional to their level of kinematics. The significantly different post-swim-up values for motility, velocity and ALH observed are in agreement with published research[151]. Swim-up was seen to select for higher motility and velocity while ALH was reduced, resulting in greater linearity of movement.

The correlation of pre-swim-up AS was not significant with the following pre-and post-swim-up parameters: concentration, motility, progressive motility, morphology, VAP,VSL, VCL and AI (Table 7). Post-swim-up motility and pre-swim-up AS showed the highest degree of correlation (Pearson r = 0.394), , however, with a r^2 value of 0.155 pre-swim-up AS was found to not predict post-swim-up motility. It was noted that there was no significant positive correlation (Table 7) between pre-swim-up AS and pre-swim-up progressive motility, VAP, VSL or VCL, while the correlation between pre-swim-up AS and post-swim-up kinematic parameters, though not

significant, becomes negative in the cases of post-swim-up progressive motility, VAP, VSL and VCL.

The reason for the negative correlations with the post-swim-up kinematic variables progressive motility, VAP, VSL and VCL may be due to the greater resistance and inertia present in motile spermatozoa with larger acrosomes. The relationship may be indirect, i.e. the large average pre-swim-up AS in a population leading to large post-swim-up AS which, due to fluid resistance kinetics, in turn results in a decrease in the measured kinematic variables. This reasoning was supported by the significant negative correlations seen between post-swim-up AS and post-swim-up VAP, VSL and VCL (Table 8).

Significant negative correlations were observed between average post-swim-up AS and three post-swim-up kinematic parameters: VAP post-swim-up, VSL post-swim-up and VCL post-swim-up (Table 8). These negative correlations translate into a linear decrease in the magnitude of these measured kinematic parameters for an increase in post-swim-up AS. The r^2 values indicate that for these four parameters between 13.6% and 22.6% of the variance in the kinematic variables was due to their post-swim-up AS. Though this percentage was reasonably small, it was postulated that during the progression of spermatozoa through the swim-up medium, resistance kinetics associated with larger acrosomes translated into the observed significant decrease in the magnitude of VAP, VSL and VCL seen for populations with greater average AS.

CHAPTER 5
CONCLUSIONS

The acrosome is a vital organelle which transports components needed in the penetration of the cumulus-oocyte complex and *zona pellucida* as well as the fertilization of an oocyte. By making use of automated analysis (CASA and ASMA), the AS and kinematics of individual spermatozoa within populations could be accurately measured and the average values representing these populations obtained. The effects and relationships between these two sets of parameters were studied and from the results, it was evident that the size of the acrosome could influence the motion of the spermatozoa.

Swim-up selection is a procedure used in many laboratories as the main sperm washing technique due to the many advantagesthe technique offers, including but not limited to allowing the selection of highly motile spermatozoa for assisted reproductive procedures[154]. However this study highlights what may be an inadvertent consequence of selecting such highly motile populations of spermaotzoa.

In this study, it was found that in pre-swim-up semen samples, no significant relationship between the kinematic parameters of the population and the average AS of the pre-swim-up population existed. This was in contrast with the post-swim-up spermatozoa populations which illustrated decreasing VAP, VSL and VCL values, showing a significant negative correlation with increasing average AS in the post-swim-up populations. We believed this to be a result of the greater inertia and

resistance of larger acrosomes, reducing the net force available for propulsion of spermatozoa.

The significant inverse relationship between AS and VAP, VSL and VCL post-swim-up was a novel finding and only speculations could be made about possible applications in the treatment of sub-fertility at the time. In the case of varying AS observed for a patient diagnosed with male factor sub-fertility, it was conceivable that selecting spermatozoa for assisted reproductive treatment on the basis of not only kinematic characteristics but also AS may increase the fertilization potential and in turn, the efficacy of treatment. It was postulated that increased efficacy of treatment may result from retaining the important functions of the acrosome due to the acrosomal contents being retained in adequate volumes, in addition to adequate kinematic properties being selected for. This contrasts with the current practice of selecting a spermatozoan population for treatment solely on the basis of kinematic properties.

Despite the continued development of automated analysis hardware and software and the great increase in processing power of computer systems in the last ten years, ASMA systems may currently fail to identify severely abnormal spermatozoa as spermatozoa. It was hoped that the development of hardware and software seen in industry would result in a continually improving detection ability and degree of classification accuracy by ASMA systems.

During swim-up, selection on the basis of kinematics was observed, higher kinematics corresponding to increased selection and thus an inadvertent selection

against acrosome size. It is also recommended that future research on this subject investigate the relationship between average AS and fertilization potential, and subsequently the merits of selecting for optimal AS to attain improved fertilization potential.

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